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**(54) Title:** MULTIPLE ADMINISTRATIONS OF ANTI-TNF ANTIBODY

**(57) Abstract**

The invention relates to a method of treating an individual having a TNF-mediated disease comprising administering to the individual multiple doses of an anti-TNF antibody wherein the second or subsequent dose is administered during or immediately prior to relapse of the disease. Preferably, the disease is a TNF $\alpha$ -mediated disease, such as rheumatoid arthritis. The anti-TNF antibody can be a monoclonal antibody or fragment thereof, such as a murine antibody, chimeric antibody or a humanized antibody or fragment thereof. Preferably, the antibody binds to one or more amino acids of human TNF $\alpha$  (hTNF $\alpha$ ) selected from the group consisting of 87-108 and 59-80. The antibody can bind to the epitope of A2 or cA2. In a preferred embodiment, the antibody is A2 or cA2.

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MULTIPLE ADMINISTRATIONS OF ANTI-TNF ANTIBODYBackground of the Invention

Monocytes and macrophages secrete a cytokine known as tumor necrosis factor- $\alpha$  (TNF $\alpha$  or TNF) in response to 5 endotoxin or other stimuli. TNF $\alpha$  is a soluble homotrimer of 17 kD protein subunits (Smith, et al., *J. Biol. Chem.* 262:6951- 6954 (1987)). A membrane-bound 26 Kd precursor form of TNF also exists (Kriegler, et al., *Cell* 53:45-53 (1988)). For reviews of TNF, see Beutler, et al., *Nature* 10 320:584 (1986), Old, *Science* 230:630 (1986), and Le, et al., *Lab. Invest.* 56:234.

Cells other than monocytes or macrophages also make TNF $\alpha$ . For example, human non-monocytic tumor cell lines produce TNF (Rubin, et al., *J. Exp. Med.* 164:1350 (1986); 15 Spriggs, et al., *Proc. Natl. Acad. Sci. USA* 84:6563 (1987)). CD4+ and CD8+ peripheral blood T lymphocytes and some cultured T and B cell lines (Cuturi, et al., *J. Exp. Med.* 165:1581 (1987); Sung, et al., *J. Exp. Med.* 168:1539 (1988)) also produce TNF $\alpha$ .

20 TNF causes pro-inflammatory actions which result in tissue injury, such as inducing procoagulant activity on vascular endothelial cells (Pober, et al., *J. Immunol.* 136:1680 (1986)), increasing the adherence of neutrophils and lymphocytes (Pober, et al., *J. Immunol.* 138:3319 25 (1987)), and stimulating the release of platelet activating factor from macrophages, neutrophils and vascular endothelial cells (Camussi, et al., *J. Exp. Med.* 166:1390 (1987)).

Thus, TNF $\alpha$  has been implicated in inflammatory 30 diseases, autoimmune diseases, viral, bacterial and parasitic infections, malignancies, and/or neurodegenerative diseases and is a useful target for specific biological therapy in diseases, such as rheumatoid arthritis and

-2-

Crohn's disease. Beneficial effects in an open-label trial with a chimeric antibody to TNF $\alpha$  (cA2) have been reported with suppression of inflammation. Elliott et al., *Arthritis Rheum* 36:1681-1690 (1993). However, patients treated with 5 the anti-TNF $\alpha$  antibodies frequently relapsed.

To date, the multiple administration of antibodies has been problematic because of the human anti-mouse antibody response (HAMA response). Exley et al., *Lancet* 335:1275-1277 (1990). Furthermore, subsequent administrations of 10 chimeric antibodies have resulted in decreased therapeutic benefit in the patient. For example, anti-TNF murine mAb therapy in fourteen patients with severe septic shock were administered a murine anti-TNF mAb in a single dose from 0.4-10 mg/kg (Exley, A.R. et al., *Lancet* 335:1275-1277 15 (1990)). However, seven of the fourteen patients developed a human anti-murine antibody response to the treatment, which treatment suffers from the problems due to immunogenicity of murine heavy and light chain portions of the antibody. Immunogenicity causes decreased effectiveness 20 of continued administration and can render treatment ineffective in patients undergoing diagnostic or therapeutic administration of murine anti-TNF antibodies. Although chimerizing such antibodies was hoped to alleviate this negative response (Morrison, *Science*, 229:1202-1207 (1985)), 25 some chimeric antibodies have proven to result in an HACA (human anti-chimeric antibody) response which at times is as great as the corresponding murine antibody. (For example, Khazaeli et al., *Cancer Res.*, 51: 5461-5466 (1991)). Even where reduced immunogenicity was observed in chimerizing 30 murine antibodies the HACA response raised still frequently precludes their use as a repeat therapy.

As such, the ability to administer an antibody in a TNF-mediated disease to treat reoccurrence of the disease state would be very beneficial.

-3-

Summary of the Invention

The invention is based on the unexpected and surprising discovery that certain anti-TNF antibodies can be administered to patients suffering from relapse of a TNF-mediated disease with a magnitude of response that was the same or similar to that achieved with the initial treatment of the antibody.

The invention, therefore, relates to a method of treating an individual having a TNF-mediated disease comprising administering to the individual multiple doses of an anti-TNF antibody wherein the second or subsequent dose is administered during or immediately prior to reoccurrence of the disease. The invention also relates to the use of anti-TNF antibodies for the treatment of reoccurrence of a TNF-mediated disease previously treated with an anti-TNF antibody. TNF-mediated diseases include chronic and debilitating disease states such as rheumatoid arthritis and Crohn's disease.

The invention permits the extended beneficial treatment of patients suffering from these chronic disease states.

Brief Description of the Drawings

Figure 1 is a line graph of the response magnitude of patients administered with a first cycle of 20 mg/kg and three cycles of 10 mg/kg of anti-TNF $\alpha$  antibody (cA2). The figure shows that the second and subsequent administrations resulted in a response that equalled or surpassed that of the first treatment cycle. The solid line indicates the swollen joint count and the dotted line indicates the serum C-reactive protein (CRP) response.

Figure 2 is a bar graph of the duration of response of seven patients administered with at least two full cycles of anti-TNF $\alpha$  antibody (cA2).

-4-

Detailed Description of the Invention

The invention relates to the discovery that anti-TNF antibodies can be administered to patients suffering from a TNF-mediated disease in multiple doses, even during relapse, 5 with good to excellent alleviation of the symptoms of the disease in the second or subsequent administration. The present invention also provides for anti-TNF antibodies in the form of pharmaceutical compositions, useful for therapeutic methods of the present invention for treating 10 TNF-related pathologies.

It is preferred to use high affinity and/or potent *in vivo* TNF-inhibiting and/or neutralizing antibodies, fragments or regions thereof. Such antibodies, fragments, or regions, will preferably have an affinity for hTNF $\alpha$ , 15 expressed as  $K_a$ , of at least  $10^8 M^{-1}$ , more preferably, at least  $10^9 M^{-1}$ .

Preferred for human therapeutic use are high affinity antibodies, and fragments, regions and derivatives having potent *in vivo* TNF $\alpha$ -inhibiting and/or neutralizing activity, 20 according to the present invention, that block TNF-induced IL-6 secretion. Also preferred for human therapeutic uses are such high affinity anti-TNF $\alpha$  antibodies, and fragments, regions and derivatives thereof, that block TNF-induced procoagulant activity, including blocking of TNF-induced 25 expression of cell adhesion molecules such as ELAM-I and ICAM-I and blocking of TNF mitogenic activity, *in vivo* and *in vitro*.

Anti-TNF antibodies which can be used in the invention include monoclonal, chimeric, humanized, resurfaced or 30 recombinant antibodies or fragments thereof which are characterized by high affinity to TNF and low toxicity (including HAMA and/or HACA response). In particular, it is preferable to employ an antibody wherein the individual components, such as the variable region, constant region and 35 framework, individually and/or collectively possess low

-5-

immunogenicity. The antibodies of the present invention are characterized by their ability to treat patients for extended periods with good to excellent alleviation of symptoms and low toxicity. Low immunogenicity and/or high 5 affinity, as well as other undefined properties, may contribute to the therapeutic results achieved. Preferred antibodies are chimeric antibodies.

Chimeric antibodies are immunoglobulin molecules characterized by two or more segments or portions derived 10 from different animal species. Generally, the variable region of the chimeric antibody is derived from a non-human mammalian antibody, such as a murine mAb, and the immunoglobulin constant region is derived from a human immunoglobulin molecule. Preferably, a variable region with 15 low immunogenicity is selected and combined with a human constant region which also has low immunogenicity, the combination also preferably having low immunogenicity.

"Low" immunogenicity is defined herein as raising significant HACA or HAMA responses in less than about 75%, 20 or preferably less than about 50% of the patients treated and/or raising low titres in the patient treated (less than about 300, preferably less than about 100 measured with a double antigen enzyme immunoassay). Elliott et al., *Lancet* 344:1125-1127 (1994), incorporated herein by reference.

25 As used herein, the term "chimeric antibody" includes monovalent, divalent or polyvalent immunoglobulins. A monovalent chimeric antibody is a dimer (HL) formed by a chimeric H chain associated through disulfide bridges with a chimeric L chain. A divalent chimeric antibody is a 30 tetramer (H2L2) formed by two HL dimers associated through at least one disulfide bridge. A polyvalent chimeric antibody can also be produced, for example, by employing a CH region that aggregates (e.g., from an IgM H chain, or  $\mu$  chain).

-6-

Antibodies comprise individual heavy (H) and/or light (L) immunoglobulin chains. A chimeric H chain comprises an antigen binding region derived from the H chain of a non-human antibody specific for TNF, which is linked to at 5 least a portion of a human H chain C region (CH), such as CH1 or CH2. A chimeric L chain according to the present invention, comprises an antigen binding region derived from the L chain of a non-human antibody specific for TNF, linked to at least a portion of a human L chain C region (CL).

10 Chimeric antibodies and methods for their production have been described in the art (Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984); Boulianne et al., *Nature* 312:643-646 (1984); Neuberger et al., *Nature* 314:268-270 (1985); Taniguchi et al., European Patent Application 171496 (published February 19, 1985); Morrison et al., European Patent Application 173494 (published March 5, 1986); Neuberger et al., PCT Application WO 86/01533, (published March 13, 1986); Kudo et al., European Patent Application 184187 (published June 11, 1986); Morrison et al., European 15 Patent Application 173494 (published March 5, 1986); Sahagan et al., *J. Immunol.* 137:1066-1074 (1986); Robinson et al., International Patent Publication #PCT/US86/02269 (published 20 7 May 1987); Liu et al., *Proc. Natl. Acad. Sci. USA* 84:3439-3443 (1987); Sun et al., *Proc. Natl. Acad. Sci. USA* 84:214-218 (1987); Better et al., *Science* 240:1041-1043 (1988); and Harlow and Lane *ANTIBODIES: A LABORATORY MANUAL* Cold Spring Harbor Laboratory (1988)). These references are 25 entirely incorporated herein by reference.

The anti-TNF chimeric antibody can comprise, for 30 example, two light chains and two heavy chains, each of the chains comprising at least part of a human constant region and at least part of a variable (V) region of non-human origin having specificity to human TNF, said antibody binding with high affinity to an inhibiting and/or 35 neutralizing epitope of human TNF, such as the antibody cA2.

-7-

The antibody also includes a fragment or a derivative of such an antibody, such as one or more portions of the antibody chain, such as the heavy chain constant or variable regions, or the light chain constant or variable regions.

5 Humanizing and resurfacing the antibody can further reduce the immunogenicity of the antibody. See, for example, Winter (U.S. Patent No. 5,225,539 and EP 239,400 B1), Padlan et al. (EP 519,596 A1) and Pedersen et al. (EP 592,106 A1) incorporated herein by reference.

10 Preferred antibodies of the present invention are high affinity human-murine chimeric anti-TNF antibodies, and fragments or regions thereof, that have potent inhibiting and/or neutralizing activity *in vivo* against human TNF $\alpha$ . Such antibodies and chimeric antibodies can include those  
15 generated by immunization using purified recombinant TNF $\alpha$  or peptide fragments thereof comprising one or more epitopes. An example of such a chimeric antibody is cA2 and antibodies which will competitively inhibit *in vivo* the binding to human TNF $\alpha$  of anti-TNF $\alpha$  murine mAb A2, chimeric mAb cA2, or  
20 an antibody having substantially the same specific binding characteristics, as well as fragments and regions thereof. Preferred methods for determining mAb specificity and affinity by competitive inhibition can be found in Harlow, et al., *Antibodies: A Laboratory Manual*, Cold Spring Harbor  
25 Laboratory Press, Cold Spring Harbor, NY, 1988), Colligan et al., eds., *Current Protocols in Immunology*, Greene Publishing Assoc. and Wiley Interscience, N.Y., (1992, 1993), Kozbor et al., *Immunol. Today* 4:72-79 (1983), Ausubel et al., eds. *Current Protocols in Molecular Biology*, Wiley  
30 Interscience, N.Y. (1987, 1992, 1993); and Muller, *Meth. Enzymol.* 92:589-601 (1983), which references are entirely incorporated herein by reference.

As used herein, the term "antigen binding region" refers to that portion of an antibody molecule which  
35 contains the amino acid residues that interact with an

antigen and confer on the antibody its specificity and affinity for the antigen. The antibody region includes the "framework" amino acid residues necessary to maintain the proper conformation of the antigen-binding residues.

5 Generally, the antigen binding region will be of murine origin. In other embodiments, the antigen binding region can be derived from other animal species, such as sheep, rabbit, rat or hamster. Preferred sources for the DNA encoding such a non-human antibody include cell lines which 10 produce antibody, preferably hybrid cell lines commonly known as hybridomas. A preferred hybridoma is the A2 hybridoma cell line.

An "antigen" is a molecule or a portion of a molecule capable of being bound by an antibody which is additionally 15 capable of inducing an animal to produce antibody capable of selectively binding to an epitope of that antigen. An antigen can have one or more than one epitope.

The term "epitope" is meant to refer to that portion of the antigen capable of being recognized by and bound by an 20 antibody at one or more of the Ab's antigen binding region. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and have specific three dimensional structural characteristics as well as specific charge characteristics. 25 By "inhibiting and/or neutralizing epitope" is intended an epitope, which, when bound by an antibody, results in loss of biological activity of the molecule containing the epitope, *in vivo* or *in vitro*, more preferably *in vivo*, including binding of TNF to a TNF receptor. Epitopes of TNF 30 have been identified within amino acids 1 to about 20, about 56 to about 77, about 108 to about 127 and about 138 to about 149. Preferably, the antibody binds to an epitope comprising at least about 5 amino acids of TNF within TNF residues from about 87 to about 107, about 59 to about 80 or 35 a combination thereof. Generally, epitopes include at least

-9-

about 5 amino acids and less than about 22 amino acids embracing or overlapping one or more of these regions.

For example, epitopes of TNF which are recognized by, and/or binds with anti-TNF activity, an antibody, and 5 fragments, and variable regions thereof, include:

59-80: Tyr-Ser-Gln-Val-Leu-Phe-Lys-Gly-Gln-Gly-  
Cys-Pro-Ser-Thr-His-Val-Leu-Leu-Thr-His-  
Thr-Ile (SEQ ID NO:1); and/or  
87-108: Tyr-Gln-Thr-Lys-Val-Asn-Leu-Leu-Ser-Ala-  
10 Ile-Lys-Ser-Pro-Cys-Gln-Arg-Glu-Thr-Pro-  
Glu-Gly (SEQ ID NO:2).

These preferred anti-TNF antibodies or peptides block the action of TNF $\alpha$  without binding to the putative receptor binding locus as presented by Eck and Sprang (J. Biol. Chem. 15 264(29): 17595-17605 (1989) (amino acids 11-13, 37-42, 49-57 and 155-157 of hTNF $\alpha$ ).

#### Antibody Production Using Hybridomas

The techniques to raise antibodies to small peptide sequences that recognize and bind to those sequences in the 20 free or conjugated form or when presented as a native sequence in the context of a large protein are well known in the art. Such antibodies can be produced by hybridoma or recombinant techniques known in the art.

Murine antibodies which can be used in the preparation 25 of the antibodies of the present invention have also been described in Rubin et al., EP0218868, April 22, 1987; Yone et al., EP0288088, October 26, 1988; Liang, et al., Biochem. Biophys. Res. Comm. 137:847-854 (1986); Meager, et al., Hybridoma 6:305-311 (1987); Fendly et al., Hybridoma 30 6:359-369 (1987); Bringman, et al., Hybridoma 6:489-507 (1987); Hirai, et al., J. Immunol. Meth. 96:57-62 (1987); Moller, et al., (Cytokine 2:162-169 (1990).

-10-

The cell fusions are accomplished by standard procedures well known to those skilled in the field of immunology. Fusion partner cell lines and methods for fusing and selecting hybridomas and screening for mAbs are 5 well known in the art. See, e.g., Ausubel *infra*, Harlow *infra*, and Colligan *infra*, the contents of which references are incorporated entirely herein by reference.

The TNF $\alpha$ -specific murine mAb of the present invention can be produced in large quantities by injecting hybridoma 10 or transfectoma cells secreting the antibody into the peritoneal cavity of mice and, after appropriate time, harvesting the ascites fluid which contains a high titer of the mAb, and isolating the mAb therefrom. For such *in vivo* production of the mAb with a hybridoma (e.g., rat or human), 15 hybridoma cells are preferably grown in irradiated or athymic nude mice. Alternatively, the antibodies can be produced by culturing hybridoma or transfectoma cells *in vitro* and isolating secreted mAb from the cell culture medium or recombinantly, in eukaryotic or prokaryotic cells.

20 In a preferred embodiment, the antibody is a mAb which binds amino acids of an epitope of TNF recognized by A2, rA2 or cA2, which is produced by a hybridoma or by a recombinant host. In another preferred embodiment, the antibody is a chimeric antibody which recognizes an epitope recognized by 25 A2. In a more preferred embodiment, the antibody is a chimeric antibody designated as chimeric A2 (cA2).

As examples of antibodies according to the present 30 invention, murine mAb A2 of the present invention is produced by a cell line designated c134A. Chimeric antibody cA2 is produced by a cell line designated c168A. c168A was deposited at the American Type Culture Collection, Rockville, Maryland, as a "Culture Safe Deposit."

The invention also provides for "derivatives" of the 35 antibodies including fragments, regions or proteins encoded by truncated or modified genes to yield molecular species

-11-

functionally resembling the immunoglobulin fragments. The modifications include, but are not limited to, addition of genetic sequences coding for cytotoxic proteins such as plant and bacterial toxins. The fragments and derivatives 5 can be produced from appropriate cells, as is known in the art. Alternatively, anti-TNF antibodies, fragments and regions can be bound to cytotoxic proteins or compounds *in vitro*, to provide cytotoxic anti-TNF antibodies which would selectively kill cells having TNF on their surface.

10 Fragments include, for example, Fab, Fab', F(ab')2 and Fv. These fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and can have less non-specific tissue binding than an intact antibody (Wahl et al., *J. Nucl. Med.* 24:316-325 (1983)). 15 These fragments are produced from intact antibodies using methods well known in the art, for example by proteolytic cleavage with enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')2 fragments).

#### Recombinant Expression of Anti-TNF Antibodies

20 Recombinant and/or chimeric murine-human or human-human antibodies that inhibit TNF can be provided according to the present invention using known techniques based on the teaching provided herein. See, e.g., Ausubel et al., eds. *Current Protocols in Molecular Biology*, Wiley Interscience, 25 N.Y. (1987, 1992, 1993); and Sambrook et al. *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (1989), the contents of which are incorporated herein by reference.

The DNA encoding an anti-TNF antibody of the present 30 invention can be genomic DNA or cDNA which encodes at least one of the heavy chain constant region (Hc), the heavy chain variable region (Hc), the light chain variable region (Lv) and the light chain constant regions (Lc). A convenient alternative to the use of chromosomal gene fragments as the

-12-

source of DNA encoding the murine V region antigen-binding segment is the use of cDNA for the construction of chimeric immunoglobulin genes, e.g., as reported by Liu et al. (Proc. Natl. Acad. Sci., USA 84:3439 (1987) and J. Immunology 5 139:3521 (1987), which references are hereby entirely incorporated herein by reference. The use of cDNA requires that gene expression elements appropriate for the host cell be combined with the gene in order to achieve synthesis of the desired protein. The use of cDNA sequences is 10 advantageous over genomic sequences (which contain introns), in that cDNA sequences can be expressed in bacteria or other hosts which lack appropriate RNA splicing systems. An example of such a preparation is set forth below.

Because the genetic code is degenerate, more than one 15 codon can be used to encode a particular amino acid. Using the genetic code, one or more different oligonucleotides can be identified, each of which would be capable of encoding the amino acid. The probability that a particular oligonucleotide will, in fact, constitute the actual XXX- 20 encoding sequence can be estimated by considering abnormal base pairing relationships and the frequency with which a particular codon is actually used (to encode a particular amino acid) in eukaryotic or prokaryotic cells expressing an anti-TNF antibody or fragment. Such "codon usage rules" are 25 disclosed by Lathe, et al., J. Molec. Biol. 183:1-12 (1985). Using the "codon usage rules" of Lathe, a single oligonucleotide, or a set of oligonucleotides, that contains 30 a theoretical "most probable" nucleotide sequence capable of encoding anti-TNF variable or constant region sequences is identified.

Although occasionally an amino acid sequence can be encoded by only a single oligonucleotide, frequently the amino acid sequence can be encoded by any of a set of similar oligonucleotides. Importantly, whereas all of the 35 members of this set contain oligonucleotides which are

-13-

capable of encoding the peptide fragment and, thus, potentially contain the same oligonucleotide sequence as the gene which encodes the peptide fragment, only one member of the set contains the nucleotide sequence that is identical 5 to the nucleotide sequence of the gene. Because this member is present within the set, and is capable of hybridizing to DNA even in the presence of the other members of the set, it is possible to employ the unfractionated set of oligonucleotides in the same manner in which one would 10 employ a single oligonucleotide to clone the gene that encodes the protein.

The oligonucleotide, or set of oligonucleotides, containing the theoretical "most probable" sequence capable of encoding an anti-TNF antibody or fragment including a 15 variable or constant region is used to identify the sequence of a complementary oligonucleotide or set of oligonucleotides which is capable of hybridizing to the "most probable" sequence, or set of sequences. An oligonucleotide containing such a complementary sequence can 20 be employed as a probe to identify and isolate the variable or constant region anti-TNF gene (Sambrook et al., *infra*).

A suitable oligonucleotide, or set of oligonucleotides, which is capable of encoding a fragment of the variable or constant anti-TNF region (or which is complementary to such 25 an oligonucleotide, or set of oligonucleotides) is identified (using the above-described procedure), synthesized, and hybridized by means well known in the art, against a DNA or, more preferably, a cDNA preparation derived from cells which are capable of expressing anti-TNF 30 antibodies or variable or constant regions thereof. Single stranded oligonucleotide molecules complementary to the "most probable" variable or constant anti-TNF region peptide coding sequences can be synthesized using procedures which are well known to those of ordinary skill in the art 35 (Belagaje, et al., *J. Biol. Chem.* 254:5765-5780 (1979);

-14-

Maniatis, et al., *In: Molecular Mechanisms in the Control of Gene Expression*, Nierlich, et al., Eds., Acad. Press, NY (1976); Wu, et al., *Prog. Nucl. Acid Res. Molec. Biol.* 21:101-141 (1978); Khorana, *Science* 203:614-625 (1979)).

5 Additionally, DNA synthesis can be achieved through the use of automated synthesizers. Techniques of nucleic acid hybridization are disclosed by Sambrook et al. (*infra*), and by Haynes, et al. (*In: Nucleic Acid Hybridization, A Practical Approach*, IRL Press, Washington, DC (1985)), which

10 references are herein incorporated by reference. Techniques such as, or similar to, those described above have successfully enabled the cloning of genes for human aldehyde dehydrogenases (Hsu, et al., *Proc. Natl. Acad. Sci. USA* 82:3771-3775 (1985)), fibronectin (Suzuki, et al., *Bur. Mol. Biol. Organ. J.* 4:2519-2524 (1985)), the human estrogen receptor gene (Walter, et al., *Proc. Natl. Acad. Sci. USA* 82:7889-7893 (1985)), tissue-type plasminogen activator (Pennica, et al., *Nature* 301:214-221 (1983)) and human placental alkaline phosphatase complementary DNA (Keun, et al., *Proc. Natl. Acad. Sci. USA* 82:8715-8719 (1985)).

15 In an alternative way of cloning a polynucleotide encoding an anti-TNF variable or constant region, a library of expression vectors is prepared by cloning DNA or, more preferably, cDNA (from a cell capable of expressing an anti-

20 TNF antibody or variable or constant region) into an expression vector. The library is then screened for members capable of expressing a protein which competitively inhibits the binding of an anti-TNF antibody, such as A2 or cA2, and which has a nucleotide sequence that is capable of encoding

25 polypeptides that have the same amino acid sequence as anti-TNF antibodies or fragments thereof. In this embodiment, DNA, or more preferably cDNA, is extracted and purified from a cell which is capable of expressing an anti-TNF antibody or fragment. The purified cDNA is

30 fragmentized (by shearing, endonuclease digestion, etc.) to

35

-15-

produce a pool of DNA or cDNA fragments. DNA or cDNA fragments from this pool are then cloned into an expression vector in order to produce a genomic library of expression vectors whose members each contain a unique cloned DNA or 5 cDNA fragment such as in a lambda phage library, expression in prokaryotic cell (e.g., bacteria) or eukaryotic cells, (e.g., mammalian, yeast, insect or, fungus). See, e.g., Ausubel, *infra*, Harlow, *infra*, Colligan, *infra*; Nyssonnen et al. *Bio/Technology* 11:591-595 (1993); Marks et al., 10 *Bio/Technology* 11:1145-1149 (October 1993). Once nucleic acid encoding such variable or constant anti-TNF regions is isolated, the nucleic acid can be appropriately expressed in a host cell, along with other constant or variable heavy or light chain encoding nucleic acid, in order to provide 15 recombinant mabs that bind TNF with inhibitory activity. Such antibodies preferably include a murine or human anti-TNF variable region which contains a framework residue having complementarity determining residues which are responsible for antigen binding.

20 Human genes which encode the constant (C) regions of the chimeric antibodies, fragments and regions of the present invention can be derived from a human fetal liver library, by known methods. Human C region genes can be derived from any human cell including those which express 25 and produce human immunoglobulins. The human CH region can be derived from any of the known classes or isotypes of human H chains, including gamma,  $\mu$ ,  $\alpha$ ,  $\delta$  or  $\epsilon$ , and subtypes thereof, such as G1, G2, G3 and G4. Since the H chain isotype is responsible for the various effector functions of 30 an antibody, the choice of CH region will be guided by the desired effector functions, such as complement fixation, or activity in antibody-dependent cellular cytotoxicity (ADCC). Preferably, the CH region is derived from gamma 1 (IgG1), gamma 3 (IgG3), gamma 4 (IgG4), or  $\mu$  (IgM). The human CL

-16-

region can be derived from either human L chain isotype, kappa or lambda.

Genes encoding human immunoglobulin C regions are obtained from human cells by standard cloning techniques 5 (Sambrook, et al. (*Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989) and Ausubel et al., eds. *Current Protocols in Molecular Biology* (1987-1993))). Human C region genes are readily available from known clones containing genes 10 representing the two classes of L chains, the five classes of H chains and subclasses thereof. Chimeric antibody fragments, such as F(ab')2 and Fab, can be prepared by designing a chimeric H chain gene which is appropriately truncated. For example, a chimeric gene encoding an H chain 15 portion of an F(ab')2 fragment would include DNA sequences encoding the CH1 domain and hinge region of the H chain, followed by a translational stop codon to yield the truncated molecule.

Generally, the murine, human and chimeric antibodies, 20 fragments and regions are produced by cloning DNA segments encoding the H and L chain antigen-binding regions of a TNF-specific antibody, and joining these DNA segments to DNA segments encoding CH and CL regions, respectively, to produce murine, human or chimeric immunoglobulin-encoding 25 genes. Thus, in a preferred embodiment, a fused chimeric gene is created which comprises a first DNA segment that encodes at least the antigen-binding region of non-human origin, such as a functionally rearranged V region with joining (J) segment, linked to a second DNA segment 30 encoding at least a part of a human C region.

Therefore, cDNA encoding the antibody V and C regions and the method of producing a chimeric antibody can involve several steps, outlined below:

-17-

1. isolation of messenger RNA (mRNA) from the cell line producing an anti-TNF antibody and from optional additional antibodies supplying heavy and light constant regions; cloning and cDNA production therefrom;
- 5
2. preparation of a full length cDNA library from purified mRNA from which the appropriate V and/or C region gene segments of the L and H chain genes can be: (i) identified with appropriate probes, (ii) sequenced, and (iii) made compatible with a C or V gene segment from another antibody for a chimeric antibody;
- 10
3. Construction of complete H or L chain coding sequences by linkage of the cloned specific V region gene segments to cloned C region gene, as described above;
- 15
4. Expression and production of L and H chains in selected hosts, including prokaryotic and eukaryotic cells to provide murine-murine, human-murine, human-human or human-murine antibodies.
- 20

One common feature of all immunoglobulin H and L chain genes and their encoded mRNAs is the J region. H and L chain J regions have different sequences, but a high degree 25 of sequence homology exists (greater than 80%) among each group, especially near the C region. This homology is exploited in this method and consensus sequences of H and L chain J regions can be used to design oligonucleotides for use as primers for introducing useful restriction sites into 30 the J region for subsequent linkage of V region segments to human C region segments.

-18-

C region cDNA vectors prepared from human cells can be modified by site-directed mutagenesis to place a restriction site at the analogous position in the human sequence. For example, one can clone the complete human kappa chain C (C<sub>k</sub>) 5 region and the complete human gamma-1 C region (C<sub>gamma</sub>-1). In this case, the alternative method based upon genomic C region clones as the source for C region vectors would not allow these genes to be expressed in bacterial systems where enzymes needed to remove intervening sequences are absent. 10 Cloned V region segments are excised and ligated to L or H chain C region vectors. Alternatively, the human C<sub>gamma</sub>-1 region can be modified by introducing a termination codon thereby generating a gene sequence which encodes the H chain portion of an Fab molecule. The coding sequences with 15 linked V and C regions are then transferred into appropriate expression vehicles for expression in appropriate hosts, prokaryotic or eukaryotic.

Two coding DNA sequences are said to be "operably linked" if the linkage results in a continuously 20 translatable sequence without alteration or interruption of the triplet reading frame. A DNA coding sequence is operably linked to a gene expression element if the linkage results in the proper function of that gene expression element to result in expression of the coding sequence.

25 Expression vehicles include plasmids or other vectors. Preferred among these are vehicles carrying a functionally complete human CH or CL chain sequence having appropriate restriction sites engineered so that any VH or VL chain sequence with appropriate cohesive ends can be easily 30 inserted therein. Human CH or CL chain sequence-containing vehicles thus serve as intermediates for the expression of any desired complete H or L chain in any appropriate host.

35 A chimeric antibody, such as a mouse-human or human-human, will typically be synthesized from genes driven by the chromosomal gene promoters native to the mouse H and

-19-

L chain V regions used in the constructs; splicing usually occurs between the splice donor site in the mouse J region and the splice acceptor site preceding the human C region and also at the splice regions that occur within the human 5 C, region; polyadenylation and transcription termination occur at native chromosomal sites downstream of the human coding regions.

A nucleic acid sequence encoding at least one anti-TNF antibody fragment may be recombined with vector DNA in 10 accordance with conventional techniques, including blunt-ended or staggered-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and 15 ligation with appropriate ligases. Techniques for such manipulations are disclosed, e.g., by Ausubel, *infra*, Sambrook, *infra*, entirely incorporated herein by reference, and are well known in the art.

A nucleic acid molecule, such as DNA, is "capable of 20 expressing" a polypeptide if it contains nucleotide sequences which contain transcriptional and translational regulatory information and such sequences are "operably linked" to nucleotide sequences which encode the polypeptide. An operable linkage is a linkage in which the 25 regulatory DNA sequences and the DNA sequence sought to be expressed are connected in such a way as to permit gene expression as anti-TNF peptides or Ab fragments in recoverable amounts. The precise nature of the regulatory regions needed for gene expression may vary from organism to 30 organism, as is well known in the analogous art. See, e.g., Sambrook, *supra* and Ausubel *supra*.

Preferred hosts are bacterial or eukaryotic hosts including bacteria, yeast, insects, fungi, bird and mammalian cells either *in vivo*, or *in situ*, or host cells of 35 mammalian, insect, bird or yeast origin. It is preferred

-20-

that the mammalian cell or tissue is of human, primate, hamster, rabbit, murine, rat, other rodent, cow, pig, sheep, horse, goat, dog or cat origin.

Further, by use of, for example, the yeast ubiquitin 5 hydrolase system, *in vivo* synthesis of ubiquitin-transmembrane polypeptide fusion proteins may be accomplished. The fusion proteins so produced may be processed *in vivo* or purified and processed *in vitro*, allowing synthesis of an anti-TNF antibody or fragment of 10 the present invention with a specified amino terminus sequence. Moreover, problems associated with retention of initiation codon-derived methionine residues in direct yeast (or bacterial) expression may be avoided. Sabin et al., *Bio/Technol.* 7(7): 705-709 (1989); Miller et al., *Bio/Technol.* 7(7):698-704 (1989).

Any of a series of yeast gene expression systems incorporating promoter and termination elements from the actively expressed genes coding for glycolytic enzymes produced in large quantities when yeast are grown in mediums 20 rich in glucose can be utilized to obtain anti-TNF antibody fragments of the present invention. Known glycolytic genes can also provide very efficient transcriptional control signals. For example, the promoter and terminator signals of the phosphoglycerate kinase gene can be utilized.

25 Production of anti-TNF antibody fragments or functional derivatives thereof in insects can be achieved, for example, by infecting the insect host with a baculovirus engineered to express transmembrane polypeptide by methods known to those of skill. See Ausubel et al., eds. *Current Protocols* 30 in *Molecular Biology* Wiley Interscience, §§16.8-16.11 (1987, 1993).

In a preferred embodiment, the introduced nucleotide sequence will be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. 35 Any of a wide variety of vectors may be employed for this

-21-

purpose. See, e.g., Ausubel et al., *infra*, §§ 1.5, 1.10, 7.1, 7.3, 8.1, 9.6, 9.7, 13.4, 16.2, 16.6, and 16.8-16.11. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells 5 that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different 10 species.

Preferred prokaryotic vectors known in the art include plasmids such as those capable of replication in E. coli (such as, for example, pBR322, ColE1, pSC101, pACYC 184, pVX). Such plasmids are, for example, disclosed by 15 Maniatis, T., et al. (*Molecular Cloning, A Laboratory Manual*, Second Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989); Ausubel, *infra*). Bacillus plasmids include pC194, pC221, pT127, etc. Such plasmids are disclosed by Gryczan, T. (In: *The Molecular Biology of the 20 Bacilli*, Academic Press, NY (1982), pp. 307-329). Suitable Streptomyces plasmids include pIJ101 (Kendall, K.J., et al., *J. Bacteriol.* 169:4177-4183 (1987)), and Streptomyces bacteriophages such as φC31 (Chater, K.F., et al., In: *Sixth International Symposium on Actinomycetales Biology*, 25 Akadémiai Kaidó, Budapest, Hungary (1986), pp. 45-54). Pseudomonas plasmids are reviewed by John, J.F., et al. (*Rev. Infect. Dis.* 8:693-704 (1986)), and Izaki, K. (*Jpn. J. Bacteriol.* 33:729-742 (1978); and Ausubel et al., *supra*).

Alternatively, gene expression elements useful for the 30 expression of cDNA encoding anti-TNF antibodies or peptides include, but are not limited to (a) viral transcription promoters and their enhancer elements, such as the SV40 early promoter (Okayama, et al., *Mol. Cell. Biol.* 3:280 (1983)), Rous sarcoma virus LTR (Gorman, et al., *Proc. Natl. Acad. Sci., USA* 79:6777 (1982)), and Moloney murine leukemia 35

-22-

virus LTR (Grosschedl, et al., *Cell* 41:885 (1985)); (b) splice regions and polyadenylation sites such as those derived from the SV40 late region (Okayarea et al., *infra*); and (c) polyadenylation sites such as in SV40 (Okayama et al., *infra*).

Immunoglobulin cDNA genes can be expressed as described by Liu et al., *infra*, and Weidle et al., *Gene* 51:21 (1987), using as expression elements the SV40 early promoter and its enhancer, the mouse immunoglobulin H chain promoter enhancers, SV40 late region mRNA splicing, rabbit S-globin intervening sequence, immunoglobulin and rabbit S-globin polyadenylation sites, and SV40 polyadenylation elements.

For immunoglobulin genes comprised of part cDNA, part genomic DNA (Whittle et al., *Protein Engineering* 1:499 (1987)), the transcriptional promoter can be human cytomegalovirus, the promoter enhancers can be cytomegalovirus and mouse/human immunoglobulin, and mRNA splicing and polyadenylation regions can be the native chromosomal immunoglobulin sequences.

In one embodiment, for expression of cDNA genes in rodent cells, the transcriptional promoter is a viral LTR sequence, the transcriptional promoter enhancers are either or both the mouse immunoglobulin heavy chain enhancer and the viral LTR enhancer, the splice region contains an intron of greater than 31 bp, and the polyadenylation and transcription termination regions are derived from the native chromosomal sequence corresponding to the immunoglobulin chain being synthesized. In other embodiments, cDNA sequences encoding other proteins are combined with the above-recited expression elements to achieve expression of the proteins in mammalian cells.

Each fused gene is assembled in, or inserted into, an expression vector. Recipient cells capable of expressing the chimeric immunoglobulin chain gene product are then transfected singly with an anti-TNF antibody or chimeric H

-23-

or chimeric L chain-encoding gene, or are co-transfected with a chimeric H and a chimeric L chain gene. The transfected recipient cells are cultured under conditions that permit expression of the incorporated genes and the 5 expressed immunoglobulin chains or intact antibodies or fragments are recovered from the culture.

In one embodiment, the fused genes encoding the anti-TNF antibody or chimeric H and L chains, or portions thereof, are assembled in separate expression vectors that 10 are then used to co-transfect a recipient cell.

Each vector can contain two selectable genes, a first selectable gene designed for selection in a bacterial system and a second selectable gene designed for selection in a eukaryotic system, wherein each vector has a different pair 15 of genes. This strategy results in vectors which first direct the production, and permit amplification, of the fused genes in a bacterial system. The genes so produced and amplified in a bacterial host are subsequently used to co-transfect a eukaryotic cell, and allow selection of a 20 co-transfected cell carrying the desired transfected genes.

Examples of selectable genes for use in a bacterial system are the gene that confers resistance to ampicillin and the gene that confers resistance to chloramphenicol. Preferred selectable genes for use in eukaryotic 25 transfectants include the xanthine guanine phosphoribosyl transferase gene (designated *gpt*) and the phosphotransferase gene from Tn5 (designated *neo*).

Selection of cells expressing *gpt* is based on the fact that the enzyme encoded by this gene utilizes xanthine as a 30 substrate for purine nucleotide synthesis, whereas the analogous endogenous enzyme cannot. In a medium containing (1) mycophenolic acid, which blocks the conversion of inosine monophosphate to xanthine monophosphate, and (2) xanthine, only cells expressing the *gpt* gene can survive. 35 The product of the *neo* blocks the inhibition of protein

-24-

synthesis by the antibiotic G418 and other antibiotics of the neomycin class.

The two selection procedures can be used simultaneously or sequentially to select for the expression of

5 immunoglobulin chain genes introduced on two different DNA vectors into a eukaryotic cell. It is not necessary to include different selectable markers for eukaryotic cells; an H and an L chain vector, each containing the same selectable marker can be co-transfected. After selection of  
10 the appropriately resistant cells, the majority of the clones will contain integrated copies of both H and L chain vectors and/or anti-TNF antibody. Alternatively, the fused genes encoding the chimeric H and L chains can be assembled on the same expression vector.

15 For transfection of the expression vectors and production of the chimeric antibody, the preferred recipient cell line is a myeloma cell. Myeloma cells can synthesize, assemble and secrete immunoglobulins encoded by transfected immunoglobulin genes and possess the mechanism for

20 glycosylation of the immunoglobulin. A particularly preferred recipient cell is the myeloma cell SP2/0 (ATCC #CRL 8287). SP2/0 cells produce only immunoglobulin encoded by the transfected genes. Myeloma cells can be grown in culture or in the peritoneal cavity of a mouse, where  
25 secreted immunoglobulin can be obtained from ascites fluid. Other suitable recipient cells include lymphoid cells such as B lymphocytes of human or non-human origin, hybridoma cells of human or non-human origin, or interspecies heterohybridoma cells.

30 The expression vector carrying a chimeric antibody construct of the present invention can be introduced into an appropriate host cell by any of a variety of suitable means, including such biochemical means as transformation, transfection, conjugation, protoplast fusion, calcium  
35 phosphate-precipitation, and application with polycations

-25-

such as diethylaminoethyl (DEAE) dextran, and such mechanical means as electroporation, direct microinjection, and microprojectile bombardment (Johnston et al., *Science* 240:1538 (1988)). A preferred way of introducing DNA into 5 lymphoid cells is by electroporation (Potter et al., *Proc. Natl. Acad. Sci. USA* 81:7161 (1984); Yoshikawa, et al., *Jpn. J. Cancer Res.* 77:1122-1133). In this procedure, recipient cells are subjected to an electric pulse in the presence of the DNA to be incorporated. Typically, after 10 transfection, cells are allowed to recover in complete medium for about 24 hours, and are then seeded in 96-well culture plates in the presence of the selective medium. G418 selection is performed using about 0.4 to 0.8 mg/ml G418. Mycophenolic acid selection utilizes about 6  $\mu$ g/ml 15 plus about 0.25 mg/ml xanthine. The electroporation technique is expected to yield transfection frequencies of about 10<sup>-5</sup> to about 10<sup>-4</sup> for Sp2/0 cells. In the protoplast fusion method, lysozyme is used to strip cell walls from catarrhal harboring the recombinant plasmid containing the 20 chimeric antibody gene. The resulting spheroplasts are fused with myeloma cells with polyethylene glycol. The immunoglobulin genes can also be expressed in nonlymphoid mammalian cells or in other eukaryotic cells, such as yeast, or in prokaryotic cells, in particular bacteria. 25 Yeast provides substantial advantages over bacteria for the production of immunoglobulin H and L chains. Yeasts carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies now exist which utilize strong promoter sequences and high copy 30 number plasmids which can be used for production of the desired proteins in yeast. Yeast recognizes leader sequences of cloned mammalian gene products and secretes peptides bearing leader sequences (i.e., pre-peptides) (Hitzman, et al., 11th International Conference on Yeast,

-26-

*Genetics and Molecular Biology, Montpellier, France,  
September 13-17, 1982).*

Yeast gene expression systems can be routinely evaluated for the levels of production, secretion and the 5 stability of anti-TNF peptides, antibody and assembled murine and chimeric antibodies, fragments and regions thereof. Yeast gene expression systems incorporating promoter and termination elements from the actively expressed genes coding for glycolytic enzymes produced in 10 large quantities when yeasts are grown in media rich in glucose, for example, can be utilized. Known glycolytic genes can also provide very efficient transcription control signals. For example, the promoter and terminator signals of the phosphoglycerate kinase (PGK) gene can be utilized. 15 A number of approaches can be taken for evaluating optimal expression plasmids for the expression of cloned immunoglobulin cDNAs in yeast (see Glover, ed., *DNA Cloning*, Vol. II, pp45-66, IRL Press, 1985).

Bacterial strains can also be utilized as hosts for the 20 production of antibody molecules or peptides described by this invention, *E. coli* K12 strains such as *E. coli* W3110 (ATCC 27325), and other enterobacteria such as *Salmonella typhimurium* or *Serratia marcescens*, and various *Pseudomonas* species can be used.

25 Plasmid vectors containing replicon and control sequences which are derived from species compatible with a host cell are used in connection with these bacterial hosts. The vector carries a replication site, as well as specific genes which are capable of providing phenotypic selection in 30 transformed cells. A number of approaches can be taken for evaluating the expression plasmids for the production of murine and chimeric antibodies, fragments and regions or antibody chains encoded by the cloned immunoglobulin cDNAs in bacteria (see Glover, ed., *DNA Cloning*, Vol. I, IRL

-27-

Press, 1985, Ausubel, *infra*, Sambrook, *infra*, Colligan, *infra*).

Preferred hosts are mammalian cells, grown *in vitro* or *in vivo*. Mammalian cells provide post-translational 5 modifications to immunoglobulin protein molecules including leader peptide removal, folding and assembly of H and L chains, glycosylation of the antibody molecules, and secretion of functional antibody protein.

Mammalian cells which can be useful as hosts for the 10 production of antibody proteins, in addition to the cells of lymphoid origin described above, include cells of fibroblast origin, such as Vero (ATCC CRL 81) or CHO-K1 (ATCC CRL 61).

Many vector systems are available for the expression of cloned anti TNF peptide H and L chain genes in mammalian 15 cells (see Glover, ed., *DNA Cloning, Vol. II*, pp143-238, IRL Press, 1985). Different approaches can be followed to obtain complete H2L2 antibodies. As discussed above, it is possible to co-express H and L chains in the same cells to achieve intracellular association and linkage of H and L 20 chains into complete tetrameric H2L2 antibodies. The co-expression can occur by using either the same or different plasmids in the same host. Genes for both H and L chains can be placed into the same plasmid, which is then transfected into cells, thereby selecting directly for cells 25 that express both chains. Alternatively, cells can be transfected first with a plasmid encoding one chain, for example the L chain, followed by transfection of the resulting cell line with an H chain plasmid containing a second selectable marker. Cell lines producing H2L2 30 molecules via either route could be transfected with plasmids encoding additional copies of peptides, H, L, or H plus L chains in conjunction with additional selectable markers to generate cell lines with enhanced properties, such as higher production of assembled H2L2 antibody

-28-

molecules or enhanced stability of the transfected cell lines.

**Chimeric A2 (cA2) Anti-TNF Antibody**

A murine-human chimeric anti-human TNF $\alpha$  MAb was 5 developed with high affinity, epitope specificity and the ability to neutralize the cytotoxic effects of human TNF. Chimeric A2 anti-TNF consists of the antigen binding variable region of the high-affinity neutralizing mouse anti-human TNF IgG1 antibody, designated A2, and the constant 10 regions of a human IgG1, kappa immunoglobulin. The human IgG1 Fc region improves allogeneic antibody effector function, increases the circulating serum half-life and decreases the immunogenicity of the antibody.

The avidity and epitope specificity of the chimeric A2 15 is derived from the variable region of the murine A2. In a solid phase ELISA, cross-competition for TNF was observed between chimeric and murine A2, indicating an identical epitope specificity of cA2 and murine A2. The specificity of cA2 for TNF- $\alpha$  was confirmed by its inability to 20 neutralize the cytotoxic effects of lymphotoxin (TNF- $\beta$ ). Chimeric A2 neutralizes the cytotoxic effect of both natural and recombinant human TNF in a dose dependent manner. From binding assays of cA2 and recombinant human TNF, the affinity constant of cA2 was calculated to be  $1.8 \times 10^9 \text{ M}^{-1}$ .

25 **Therapeutic Methods for Treating TNF-Related Pathologies**

The anti-TNF antibodies, fragments and/or derivatives are useful for treating a subject having a pathology or condition associated with abnormal levels of a substance reactive with an anti-TNF antibody, in particular TNF in 30 excess of, or less than, levels present in a normal healthy subject, where such excess or diminished levels occur in a systemic, localized or particular tissue type or location in the body. Such tissue types can include, but are not limited

-29-

to, blood, lymph, CNS, liver, kidney, spleen, heart muscle or blood vessels, brain or spinal cord white matter or grey matter, cartilage, ligaments, tendons, lung, pancreas, ovary, testes, prostate. Increased or decreased TNF

5 concentrations relative to normal levels can also be localized to specific regions or cells in the body, such as joints, nerve blood vessel junctions, bones, specific tendons or ligaments, or sites of infection, such as bacterial or viral infections.

10 TNF related pathologies or diseases include, but are not limited to, the following:

(A) acute and chronic immune and autoimmune pathologies, such as systemic lupus erythematosus (SLE), rheumatoid arthritis, thyroiditis, graft versus host

15 disease, scleroderma, diabetes mellitus, Graves' disease, and the like;

(B) infections, including, but not limited to, sepsis syndrome, cachexia, circulatory collapse and shock resulting from acute or chronic bacterial infection, acute and chronic

20 parasitic and/or infectious diseases, bacterial, viral or fungal; such as a HIV, AIDS (including symptoms of cachexia, autoimmunity disorders, AIDS dementia complex and infections);

(C) inflammatory diseases, such as chronic inflammatory pathologies and vascular inflammatory

25 pathologies, including chronic inflammatory pathologies such as sarcoidosis, chronic inflammatory bowel disease, ulcerative colitis, and Crohn's pathology and vascular inflammatory pathologies, such as, but not limited to, disseminated intravascular coagulation, atherosclerosis, and

30 Kawasaki's pathology:

(D) neurodegenerative diseases, including, but are not limited to, demyelinating diseases, such as multiple sclerosis and acute transverse myelitis; extrapyramidal and cerebellar disorders' such as lesions of the corticospinal

35 system; disorders of the basal ganglia or cerebellar

-30-

disorders; hyperkinetic movement disorders such as Huntington's Chorea and senile chorea; drug-induced movement disorders, such as those induced by drugs which block CNS dopamine receptors; hypokinetic movement disorders, such as

5 5 Parkinson's disease; Progressive supranucleo palsy; Cerebellar and Spinocerebellar Disorders, such as astructural lesions of the cerebellum; spinocerebellar degenerations (spinal ataxia, Friedreich's ataxia, cerebellar cortical degenerations, multiple systems

10 10 degenerations (Mencel, Dejerine-Thomas, Shi-Drager, and MachadoJoseph)); and systemic disorders (Refsum's disease, abetalipoproteinemia, ataxia, telangiectasia, and mitochondrial multi-system disorder); demyelinating core disorders, such as multiple sclerosis, acute transverse myelitis; disorders

15 15 of the motor unit, such as neurogenic muscular atrophies (anterior horn cell degeneration, such as amyotrophic lateral sclerosis, infantile spinal muscular atrophy and juvenile spinal muscular atrophy); Alzheimer's disease; Down's Syndrome in middle age; Diffuse Lewy body disease;

20 20 Senile Dementia of Lewy body type; Wernicke-Korsakoff syndrome; chronic alcoholism; Creutzfeldt-Jakob disease; Subacute sclerosing panencephalitis, Hallerrorden-Spatz disease; and Dementia pugilistica, or any subset thereof;

(E) malignant pathologies involving TNF-secreting

25 25 tumors or other malignancies involving TNF, such as, but not limited to leukemias (acute, chronic myelocytic, chronic lymphocytic and/or myelodyspastic syndrome); lymphomas (Hodgkin's and non-Hodgkin's lymphomas, such as malignant lymphomas (Burkitt's lymphoma or Mycosis fungoides)); and

30 30 (F) alcohol-induced hepatitis.

See, e.g., Berkow et al., eds., *The Merck Manual*, 16th edition, chapter 11, pp 1380-1529, Merck and Co., Rahway, N.J., 1992, incorporated herein by reference.

The terms "reoccurrence", "flare-up" or "relapse of the

35 35 patient" are defined to encompass the reappearance of one or

-31-

more symptoms of the disease state. For example, in the case of rheumatoid arthritis, a reoccurrence can include the experience of one or more of swollen joints, morning stiffness or joint tenderness.

5 Anti-TNF antibodies can be administered by any means that enables the active agent to reach the agent's site of action in the body of a mammal. The primary focus is the ability to reach and bind with TNF released by monocytes and macrophages or other TNF producing cells. Because proteins  
10 are subject to being digested when administered orally, parenteral administration, i. e., intravenous, subcutaneous, intramuscular, would ordinarily be used to optimize absorption.

Anti-TNF antibodies can be administered either as  
15 individual therapeutic agents or in combination with other therapeutic agents. They can be administered alone, but are generally administered with a pharmaceutical carrier selected on the basis of the chosen route of administration and standard pharmaceutical practice.

20 The dosage administered will, of course, vary depending upon known factors such as the pharmacodynamic characteristics of the particular agent, and its mode and route of administration; age, health, and weight of the recipient; nature and extent of symptoms, kind of concurrent  
25 treatment, frequency of treatment, and the effect desired. Usually a daily dosage of active ingredient can be about 0.01 to 100 milligrams per kilogram of body weight.

Ordinarily 1 to 40 milligrams per kilogram per day given in divided doses 1 to 6 times a day or in sustained release  
30 form is effective to obtain desired results. The second or subsequent administration can be administered at a dosage which is the same, less than or greater than the initial or previous dose administered to the patient.

The second or subsequent administration is preferably  
35 during or immediately prior to relapse or a flare-up of the

-32-

disease or symptoms of the disease. For example, the second and subsequent administrations can be given between about 5 to 30 weeks or about 10 to 25 weeks from the previous administration. Two, three, four or more total 5 administrations can be delivered to the patient, as needed.

Dosage forms (composition) suitable for internal administration generally contain from about 0.1 milligram to about 500 milligrams of active ingredient per unit. In these pharmaceutical compositions the active ingredient will 10 ordinarily be present in an amount of about 0.5-95% by weight based on the total weight of the composition.

For parenteral administration, anti-TNF antibodies or fragments can be formulated as a solution, suspension, emulsion or lyophilized powder in association with a 15 pharmaceutically acceptable parenteral vehicle. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Liposomes and nonaqueous vehicles such as fixed oils can also be used. The vehicle or lyophilized powder can contain additives that 20 maintain isotonicity (e.g., sodium chloride, mannitol) and chemical stability (e.g., buffers and preservatives). The formulation is sterilized by commonly used techniques.

Suitable pharmaceutical carriers are described in Remington's Pharmaceutical Sciences, A. Osol, a standard 25 reference text in this field of art.

For example, a parenteral composition suitable for administration by injection is prepared by dissolving 1.5% by weight of active ingredient in 0.9% sodium chloride solution.

30 Anti-TNF antibodies of this invention can be adapted for therapeutic efficacy by virtue of their ability to mediate antibody-dependent cellular cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC) against cells having TNF associated with their surface. For these 35 activities, either an endogenous source or an exogenous

-33-

source of effector cells (for ADCC) or complement components (for CDC) can be utilized. The murine and chimeric antibodies, fragments and regions of this invention, their fragments, and derivatives can be used therapeutically as 5 immunoconjugates (see for review: Dillman, R.O., *Ann. Int. Med.* 111:592-603 (1989)). Such peptides or Abs can be coupled to cytotoxic proteins, including, but not limited to ricin-A, *Pseudomonas* toxin and *Diphtheria* toxin. Toxins conjugated to antibodies or other ligands or peptides are 10 well known in the art (see, for example, Olsnes, S. et al., *Immunol. Today* 10:291-295 (1989)). Plant and bacterial toxins typically kill cells by disrupting the protein synthetic machinery.

Anti-TNF antibodies can be conjugated to additional 15 types of therapeutic moieties including, but not limited to, radionuclides, therapeutic agents, cytotoxic agents and drugs. Examples of radionuclides which can be coupled to antibodies and delivered *in vivo* to sites of antigen include 212Bi, 131I, 186Re, and 90Y, which list is not intended to be 20 exhaustive. The radionuclides exert their cytotoxic effect by locally irradiating the cells, leading to various intracellular lesions, as is known in the art of radiotherapy.

Cytotoxic drugs which can be conjugated to anti-TNF 25 antibodies and subsequently used for *in vivo* therapy include, but are not limited to, daunorubicin, doxorubicin, methotrexate, and Mitomycin C. Cytotoxic drugs interfere with critical cellular processes including DNA, RNA, and protein synthesis. For a description of these classes of 30 drugs which are well known in the art, and their mechanisms of action, see Goodman, et al., *Goodman and Gilman's THE PHARMACOLOGICAL BASIS OF THERAPEUTICS*, 8th Ed., Macmillan Publishing Co., 1990.

Anti-TNF antibodies can be advantageously utilized in 35 combination with other monoclonal or murine and chimeric

-34-

antibodies, fragments and regions, or with lymphokines or hemopoietic growth factors, etc., which serve to increase the number or activity of effector cells which interact with the antibodies.

- 5       Anti-TNF antibodies, fragments or derivatives can also be used in combination with TNF therapy to block undesired side effects of TNF. Recent approaches to cancer therapy have included direct administration of TNF to cancer patients or immunotherapy of cancer patients with lymphokine
- 10      activated killer (LAK) cells (Rosenberg et al., *New Eng. J. Med.* 313:1485-1492 (1985)) or tumor infiltrating lymphocytes (TIL) (Kurnick et al. (*Clin. Immunol. Immunopath.* 38:367-380 (1986); Kradin et al., *Cancer Immunol. Immunother.* 24:76-85 (1987); Kradin et al., *Transplant. Proc.* 20:336-338 (1988)).
- 15      Trials are currently underway using modified LAK cells or TIL which have been transfected with the TNF gene to produce large amounts of TNF. Such therapeutic approaches are likely to be associated with a number of undesired side effects caused by the pleiotropic actions of TNF as described herein and known in the related arts. According
- 20      to the present invention, these side effects can be reduced by concurrent treatment of a subject receiving TNF or cells producing large amounts of TIL with the antibodies, fragments or derivatives of the present invention.
- 25      Effective doses are as described above. The dose level will require adjustment according to the dose of TNF or TNF-producing cells administered, in order to block side effects without blocking the main anti-tumor effect of TNF. One of ordinary skill in the art will know how to determine
- 30      such doses without undue experimentation.

#### **Treatment of Arthritis**

In rheumatoid arthritis, the main presenting symptoms are pain, stiffness, swelling, and loss of function (Bennett JC. *The etiology of rheumatoid arthritis.* In *Textbook of*

-35-

Rheumatology (Kelley WN, Harris ED, Ruddy S, Sledge CB, eds.) WB Saunders, Philadelphia pp 879-886, 1985).

TNF $\alpha$  is of major importance in the pathogenesis of rheumatoid arthritis. Evidence for the production of TNF $\alpha$  5 is present in rheumatoid arthritis joint tissues and synovial fluid at the protein and mRNA level (Buchan G, et al., *Clin. Exp. Immunol.* 73: 449-455, 1988), indicating local synthesis. However, detecting TNF $\alpha$  in rheumatoid arthritis joints even in quantities sufficient for bioactivation does 10 not necessarily indicate that it is important in the pathogenesis of rheumatoid arthritis, nor that it is a good candidate therapeutic target. In order to address these questions, the effects of anti-TNF antibody and peptides (rabbit or monoclonal) on rheumatoid joint cell cultures, 15 and for comparison, osteoarthritic cell cultures, have been studied. IL-1 production was abolished, showing TNF $\alpha$  as a suitable therapeutic target for the therapy of rheumatoid arthritis, since anti-TNF $\alpha$  blocks both TNF and IL-1, the two cytokines known to be involved in cartilage and bone 20 destruction (Brennan et al., *Lancet* 11:244-247, 1989).

Subsequent studies in rheumatoid arthritis tissues have supported this hypothesis. Anti-TNF Abs abrogated the production of another proinflammatory cytokine, GM-CSF (Haworth et al., *Bur. J. Immunol.* 21:2575-2579, 1991). This 25 observation has been independently confirmed (Alvaro-Gracia et al, 1991). It has also been demonstrated that anti-TNF diminishes cell adhesion and HLA class II expression in rheumatoid arthritis joint cell cultures.

Having now generally described the invention, the same 30 will be further understood by reference to certain specific examples which are included herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

-36-

PREPARATION OF CA2

**EXAMPLE I: Production a Mouse Anti-Human TNF mAb**

To facilitate clinical study of TNF mAb a high-affinity potent inhibiting and/or neutralizing mouse anti-human TNF

5 IgG1 mAb designated A2 was produced.

Female BALB/c mice, 10 weeks old, were obtained from the Jackson Laboratory (Bar Harbor, ME). Forty  $\mu$ g of purified *E. coli*-derived recombinant human TNF (rhTNF) emulsified with an equal volume of complete Freund's 10 adjuvant (obtained from Difco Laboratories) in 0.4 ml was injected subcutaneously and intraperitoneally (i.p.) into a mouse. One week later, an injection of 5  $\mu$ g of rhTNF in incomplete Freund's adjuvant was given i.p. followed by four consecutive i.p. injections of 10  $\mu$ g of TNF without 15 adjuvant. Eight weeks after the last injection, the mouse was boosted i.p. with 10  $\mu$ g of TNF.

Four days later, the mouse was sacrificed, the spleen was obtained and a spleen cell suspension was prepared. Spleen cells were fused with cells of the nonsecreting 20 hybridoma, Sp2/0 (ATCC CRL1581), at a 4:1 ratio of spleen cells to Sp2/0 cells, in the presence of 0.3 ml of 30% polyethylene glycol, PEG 1450. After incubation at 37°C for 6 hours, the fused cells were distributed in 0.2 ml aliquots into 96-well plates at concentrations of  $2 \times 10^4$  SP2/0 cells 25 per well. Feeder cells, in the form of  $5 \times 10^4$  normal BALB/c spleen cells, were added to each well.

The growth medium used consisted of RPMI-1640 medium, 10% heat-inactivated fetal bovine serum (FBS) (HYCLONE), 0.1 mM minimum essential medium (MEM) nonessential amino 30 acids, 1 mM sodium pyruvate, 2mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin (GIBCO Laboratories) and, for selection, hypoxanthine-aminopterin-thymidine (HAT) (Boehringer Mannheim). A solid-phase radioimmunoassay (RIA) was employed for screening supernatants for the presence of 35 mAbs specific for rhTNF $\alpha$ . This assay is described in

-37-

Example II, below. The background binding in this assay was about 500 cpm. A supernatant was considered positive if it yielded binding of 2000 cpm or higher.

Of 322 supernatants screened, 25 were positive by RIA.

5 Of these 25, the one with the highest binding (4800 cpm) was designated A2. Positive wells were subcloned at limiting dilution on mouse feeder cells. Upon further analysis of the supernatants in neutralization assays, A2 was found to be the only positive clone showing potent inhibiting and/or 10 neutralizing activity. Thus, the hybridoma line A2 was selected. This line was maintained in RPMI-1640 medium with 10% FBS (GIBCO), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

15 Alternatively, anti-TNF antibodies which inhibit TNF biological activity can be screened by binding to peptide including at least 5 amino acids of residues 87-108 or both residues 59-80 and 87-108 of TNF or combinations of peptides contained therein, which are used in place of the rTNF 20 protein, as described above.

**EXAMPLE II: Characterization of the Anti-TNF antibody  
Radioimmunoassays**

E. coli-derived rhTNF was diluted to 1 µg/ml in BCB buffer, pH 9.6, and 0.1 ml of the solution was added to each 25 assay well. After incubation at 4°C overnight, the wells were washed briefly with BCB, then sealed with 1% bovine incubated with 40 pg/ml of natural (GENZYME, Boston, MA) or recombinant (SUNTORY, Osaka, Japan) human TNF $\alpha$  with varying concentrations of mAb A2 in the presence of 20 µg/ml 30 cycloheximide at 39°C overnight. Controls included medium alone or medium + TNF in each well. Cell death was measured by staining with naphthol blue-black, and the results read spectrophotometrically at 630 nm. Absorbance at this wave length correlates with the number of live cells present.

-38-

It was found that A2 inhibited or neutralized the cytotoxic effect of both natural and rhTNF in a dose-dependent manner.

In another experiment, the specificity of this inhibiting and/or neutralizing activity was tested. A673/6 cells were seeded at  $3 \times 10^4$  cells/well 20 hr before the TNF bioassay. Two-fold serial dilutions of rhTNF, *E. coli*-derived recombinant human lymphotoxin (TNF $\beta$ ), and *E. coli*-derived recombinant murine TNF were prepared. The A2 hybridoma supernatant was added to an equal volume of the diluted TNF preparations, and the mixtures were incubated at room temperature for 30 min. Aliquots of 0.1 ml were transferred to the wells containing A673/6 cells, 20  $\mu$ g/ml of cycloheximide was added, and the cells were incubated at 39°C overnight. The cells were then fixed and stained for evaluation of cytotoxicity. The results indicate that mAb A2 specifically inhibited or neutralized the cytotoxicity of rhTNF $\alpha$ , whereas it had no effect on human lymphotoxin (TNF $\beta$ ) or murine TNF.

Experiments were next performed to analyze the cross-reactivity of mAb A2 with TNF derived from non-human primates. Monocytes isolated from B514 (baboon), J91 (cynomolgus) and RH383 (rhesus) blood by Ficoll gradient centrifugation and adherence, were incubated at  $1 \times 10^5$  cells/well in RPMI 1640 medium with 5% FBS and 2  $\mu$ g/ml of *E. coli* LPS for 3 or 16 hr at 37°C to induce TNF production. Supernatants from duplicate wells were pooled and stored at 4°C for less than 20 hr until the TNF bioassay was performed, as described above, using A673/6 cells. Two-fold dilutions of the culture supernatants were mixed with either medium or purified mAb A2 at a final concentration of 1  $\mu$ g/ml, incubated at room temperature for 30 min and aliquots transferred to the indicator cells. The results showed that mAb A2 failed to significantly inhibit or

-39-

neutralize the cytotoxic activity of TNF produced by baboon, cynomolgus and rhesus monkey monocytes.

A further experiment was conducted with chimpanzee TNF. Monocytes isolated from CH563 (chimpanzee) blood were 5 incubated as described above to generate TNF-containing supernatants. The ability of 10 µg/ml of mAb A2 to inhibit or neutralize the bioactivity of these supernatants was assayed as above. Human TNF was used as a positive control. Results indicate that mAb A2 had potent inhibiting and/or 10 neutralizing activity for chimpanzee TNF, similar to that for human TNF.

The inhibiting and/or neutralizing activity of mAb A2 was compared with three other murine mAbs specific for human TNF, termed TNF-1, TNF-2 and TNF-3, and a control mAb. 15 Two-fold serial dilutions of purified mAbs were mixed with rhTNF (40 pg/ml), incubated at room temperature for 30 min, and aliquots tested for TNF bioactivity as above. It was found that mAbs TNF-1, TNF-2 and TNF-3 each had a similar moderate degree of inhibiting and/or neutralizing activity. 20 In contrast, mAb A2 had much more potent inhibiting and/or neutralizing activity.

**EXAMPLE III: General Strategy for Cloning Antibody V and C Genes**

The strategy for cloning the V regions for the H and L 25 chain genes from the hybridoma A2, which secretes the anti-TNF antibody described above, was based upon the linkage in the genome between the V region and the corresponding J (joining) region for functionally rearranged (and expressed) Ig genes. J region DNA probes can be used 30 to screen genomic libraries to isolate DNA linked to the J regions. Although DNA in the germline configuration (i.e., unarranged) would also hybridize to J probes, this DNA would not be linked to a Ig V region sequence and can be

-40-

identified by restriction enzyme analysis of the isolated clones.

The cloning utilized herein was to isolate V regions from rearranged H and L chain genes using  $J_H$  and  $J_k$  probes.

5 These clones were tested to see if their sequences were expressed in the A2 hybridoma by Northern analysis. Those clones that contained expressed sequence were cloned into expression vectors containing human C regions and transfected into mouse myeloma cells to determine if an

10 antibody was produced. The antibody from producing cells was then tested for binding specificity and functionally compared to the A2 murine antibody.

#### EXAMPLE IV - Construction of a L Chain Genomic Library

To isolate the L chain V region gene from the A2 hybridoma, a size-selected genomic library was constructed

15 using the phage lambda vector charon 27. High molecular weight DNA was isolated from A2 hybridoma cells and digested to completion with restriction endonuclease HindIII. The DNA was then fractionated on a 0.8% agarose gel and the DNA

20 fragments of three different size ranges of approximately 3 kb, 4 kb and 6 kb were isolated from the gel by electroelution. The size ranges for library construction were chosen based upon the size of Hind III fragments that hybridized on a southern blot with the  $J_k$  probe. After

25 phenol/chloroform extraction and ethanol precipitation, the DNA fragments from each size class were ligated with lambda charon 27 arms and packaged into phage particles *in vitro* using Gigapack Gold from Stratagene (LaJolla, CA).

These libraries were screened directly at a density of

30 approximately 20,000 plaques per 150 mm petri dish using a  $^{32}P$ -labeled  $J_k$  probe. The mouse L chain  $J_k$  probe was a 2.7 kb HindIII fragment containing all five  $J_k$  segments. The probe was labeled with  $^{32}P$  by random priming using a kit obtained from Boehringer Mannheim. Free nucleotides were

-41-

removed by centrifugation through a Sephadex G-50 column. The specific activities of the probe was approximately  $10^9$  cpm/ $\mu$ g.

5 Plaque hybridizations were carried out in 5x SSC, 50% formamide, 2x Denhardt's reagent, and 200  $\mu$ g/ml denatured salmon sperm DNA at 42°C for 18-20 hours. Final washes were in 0.5x SSC, 0.1% SDS at 65°C. Positive clones were identified after autoradiography.

**EXAMPLE V - Construction of H Chain Genomic Library**

10 To isolate the V region gens for the A2 H chain, a genomic library was constructed in the lambda gt10 vector system. High molecular weight DNA was digested to completion with restriction endonuclease EcoRI and fragments of approximately 7.5 kb were isolated after agarose gel 15 electrophoresis. These fragments were ligated with lambda gt10 arms and packaged into phage particles in vitro using Gigapack Gold.

This library was screened at a density of 20,000 plaques per 150 rnm plate using a  $J_H$  probe. The  $J_H$  probe 20 was a 2kb BamHI/EcoRI fragment containing both  $J_3$  and  $J_4$  segments. The probe was labeled as in Example III and had a similar specific radioactivity. Hybridization and wash conditions were identical to those used in Example III.

**EXAMPLE VI - Cloning of the TNF-Specific V gene regions**

25 Several positive clones were isolated from the H and L chain libraries after screening approximately  $10^6$  plaques from each library using the  $J_H$  and  $J_K$  probes, respectively. Following plaque purification, bacteriophage DNA was isolated for each positive clone, digested with either EcoRI 30 (H chain clones) or HindIII (L chain clones) and fractionated on 1% agarose gels. The DNA was transferred to

-42-

nitrocellulose and the blots were hybridized with the  $J_H$  or the  $J_k$  probe.

Several H chain clones were obtained that contained 7.5 k/D EcoRI DNA encoding fragments of MAbs to the  $J_H$  5 probe. For the light chain libraries, several clones from each of the three size-selected libraries were isolated that contained HindIII fragments that hybridize to the  $J_k$  probe. For the L chain, several independently derived HindIII fragments of 2.9 kb from the 2 kb library hybridized with a 10 1250 bp mRNA from A2, but not with SP2/0 mRNA (see Example VII). In addition, several HindIII fragments derived from the 4 kb library hybridized both to the A2 mRNA and the fusion partner mRNA. A 5.7 kb HindIII fragment from the 6 kb library did not hybridize to either RNA.

15 The observed lengths of hybridizing A2 mRNA were the correct sizes for H and L chain mRNA, respectively. Because the RNA expression was restricted to the A2 hybridoma, it was assumed that the 7.5 kb H chain fragments and the 2.9 kb L chain fragments contained the correct V region sequences 20 from A2. One example of each type was chosen for further study. The important functional test is the demonstration that these V region sequences, when combined with appropriate C region sequences, are capable of directing the synthesis of an antibody with a specificity and affinity 25 similar to that of the murine A2 antibody.

The 7.5 kb H chain fragment and the 2.9 kb L chain fragment were subcloned into plasmid vectors that allow expression of the chimeric mouse/human proteins in murine myeloma cells (see Examples VIII and IX). These plasmids 30 were co-transfected into SP2/0 cells to ascertain if intact antibody molecules were secreted, and if so, if they were of the correct specificity and affinity. Control transfections were also performed pairing the putative anti-TNF H chain with an irrelevant, but expressed, L chain; the putative 35 anti-TNF L chain was also paired with an irrelevant, but

-43-

expressed, H chain. The results indicated that the 7.5 kb H chain fragment could be expressed, whereas the 2.9 kb L chain fragment could not. This was confirmed by DNA sequence analysis that suggested portions of the coding 5 region were not in the proper amino acid reading frame when compared to other known L chain amino acid sequences.

Because the 2.9 kb HindIII fragment appeared not to contain a functional V gene, the 4.0 kb and 5.7 kb HindIII fragments isolated from L chain libraries were cloned into 10 expression vectors and tested for expression of chimeric antibody after co-transfection with the 7.5 kb H chain. The 5.7 kb HindIII fragment was incapable of supporting antibody expression, whereas the 4.0 kb HindIII fragment did support antibody expression. The antibody resulting from the 15 co-transfection of the 7.5 kb putative H chain V region and the 4.0 kb L chain V region was purified, tested in solid phase TNF binding assay, and found to be inactive. It was concluded that the V region contained on the 4.0 kb HindIII fragment was not the correct anti-TNF V regions, but was 20 contributed to the hybridoma by the fusion partner. This was subsequently confirmed by sequence analysis of cDNA derived from the A2 hybridoma and from the fusion partner.

Other independently derived L chain clones containing 2.9 kb HindIII fragments that hybridized with A2 mRNA were 25 characterized in more detail. Although the restriction maps were similar, the clones fell into two classes with respect to the presence or absence of an AccI enzyme site. The original (non-functional) 2.9 kb fragment (designated clone 8.3) was missing an AccI site present in some other clones 30 (represented by clone 4.3). The DNA sequence of clone 4.3 was extremely similar to clone 8.3, but contained a single amino acid reading frame with close homology to known L chains, unlike clone 8.3. The 2.9 kb HindIII fragment from clone 4.3 was subcloned into the L chain expression vector 35 and co-transfected with the putative anti-TNF H chain into

-44-

SP2/0 cells. An antibody was synthesized, purified and tested in the solid phase TNF binding assay. This antibody bound to TNF, and therefore, the clone 4.3 L chain V region was assumed to be the correct one.

5 The A2 murine hybridoma has been shown to contain at least four rearranged L chain V region genes. At least two of these are expressed as proteins: clone 4.3 (the correct anti-TNF L chain gene) and the gene contained in the 4.0 kb HindIII fragment (contributed by the fusion partner). The 10 expression of two L chains implies that the resulting antibody secreted from the murine hybridoma is actually a mixture of antibodies, some using the correct L chain, some using the incorrect L chain, and some using one of each. The presence of two different L chains in the murine A2 15 antibody has been confirmed by SDS gel and N-terminal protein sequence analysis of the purified antibody. Because construction of the chimeric A2 antibody involves cloning the individual H and L chain genes and expressing them in a non-producing cell line, the resulting antibody will have 20 only the correct L chain and therefore should be a more potent antibody (see Examples X, XI and XII).

#### EXAMPLE VII - Northern Analysis of Cloned DNA

Cloned DNA corresponding to the authentic H and L chain V regions from the A2 hybridoma would be expected to 25 hybridize to A2 mRNA. Non-functional DNA rearrangements at either the H or L chain genetic loci should not be expressed.

Ten  $\mu$ g total cellular RNA was subjected to 30 electrophoresis on 1% agarose/formaldehyde gels (Sambrook et al., *infra*) and transferred to nitrocellulose. Blots were hybridized with random primed DNA probes in 50% formamide, 2x Denhardt's solution, 5x SSC, and 200  $\mu$ g/ml denatured salmon sperm DNA at 42°C for 10 hours. Final wash conditions were 0.5 x SSC, 0.1% SDS at 65°C.

-45-

The subcloned DNA fragments were labeled with  $^{32}\text{P}$  by random priming and hybridized to Northern blots containing total RNA derived from A2 cells or from cells of SP2/0, the fusion partner parent of A2. The 7.5 kb EcoRI H chain 5 fragment hybridized with a 2 kb mRNA from A2, but not with SP2/0 mRNA. Similarly, the 2.9 kb L chain HindIII fragment (clone 4.3) hybridized with a 1250 bp mRNA from A2, but not with SP2/0 mRNA. The observed lengths of A2 mRNA 10 hybridizing were the correct sizes for H and L chain mRNA, respectively, confirming that the V region sequences on these DNA fragments are expressed in A2 hybridoma cells.

**EXAMPLE VIII - Construction of Expression Vectors**

The putative L (clone 4.3) and H chain V genes described above were joined to human kappa and gamma 15 constant region genes in expression vectors. The 7.5 kb EcoRI fragment corresponding to the putative  $V_H$  region gene from A2 was cloned into an expression vector containing the human  $C_{\gamma}$  gene and the Ecogpt gene to yield the plasmid designated pA2HGlapgpt.

20 The 2.9 kb putative VL fragment from clone 4.3 was cloned into a vector containing the human kappa  $C_k$  gene and the Ecogpt gene to allow selection in mammalian cells. The resulting plasmid was designated pA2HuKapgpt.

**Example IX - Expression of Chimeric Antibody Genes**

25 To express the chimeric H and L chain genes, the expression plasmids were transfected into cells of the non-producing mouse myeloma cell line, SP2/0. Plasmid DNA to be transfected was purified by centrifuging to equilibrium in ethidium bromide/cesium chloride gradients 30 twice. Plasmid DNA (10-50  $\mu\text{g}$ ) was added to  $10^7$  SP2/0 cells in medium containing Hank's salts, and the mixture was placed in a BIORAD electroporation apparatus. Electropora-

-46-

tion was performed at 20 volts, following which the cells were plated in 96 well microtiter plates.

Mycophenolic acid selection was applied after 24 hours and drug resistant colonies were identified after 1-2 weeks.

5 Resistant colonies were expanded to stable cell lines and tissue culture supernatant from these cell lines was tested for antibody using an ELISA assay with goat anti-human IgG Fc antibody and goat anti-human H+L conjugated with alkaline phosphatase (obtained from Jackson Laboratories).

10 The chimeric A2 antibody was purified from tissue culture supernatant by Protein A-Sepharose chromatography. The supernatant was adjusted to 0.1M Tris, 0.002M EDTA, pH 8.0 and loaded on a Protein A-Sepharose column equilibrated in the same buffer. The IgG was eluted with 15 0.1M citrate, pH 3.5, inhibited or neutralized with 1M Tris, and dialyzed into phosphate buffered saline (PBS).

The purified chimeric antibody was evaluated for its binding and inhibiting and/or neutralizing activity.

**EXAMPLE X - Specificity of an Anti-TNF Chimeric Antibody**

20 Since the antigen binding domain of cA2 was derived from murine A2, these mAbs would be expected to compete for the same binding site on TNF. Fixed concentrations of chimeric A2 and murine mAb A2 were incubated with increasing concentrations of murine and chimeric A2 competitor, 25 respectively, in a 96-well microtiter plate coated with rhTNF (Dainippon, Osaka, Japan). Alkaline-phosphatase conjugated anti-human immunoglobulin and anti-mouse immunoglobulin second antibodies were used to detect the level of binding of chimeric and murine A2, respectively. 30 Cross-competition for TNF antigen was observed in this solid-phase ELISA format. This finding is consistent with the expected identical epitope specificity of cA2 and murine A2.

-47-

The affinity constant for binding of mouse mAb A2 and cA2 to rhTNF $\alpha$  was determined by Scatchard analysis (see, for example, Scatchard, Ann. N.Y. Acad. Sci. 51:660 (1949)). This analysis involved measuring the direct binding of  $^{125}\text{I}$  5 labelled cA2 to iramobilized rhTNF $\alpha$  in a 96-well plate. The antibodies were each labelled to a specific activity of about  $9.7 \mu\text{Ci}/\mu\text{g}$  by the iodogen method. An affinity constant (Ka) of  $0.5 \times 10^9$  liters/mole was calculated for the mouse mAb A2. Unexpectedly, the chimeric A2 antibody 10 had a higher affinity, with a Ka of  $1.8 \times 10^9$  liters/mole. Thus, the chimeric anti-TNF $\alpha$  antibody of the present invention was shown to exhibit a significantly higher affinity of binding to human TNF $\alpha$  than did the parental murine A2 mAb. This finding was surprising, since murine 15 and chimeric antibodies, fragments and regions would be expected to have affinities that are equal to or less than that of the parent mAb.

Such high affinity anti-TNF antibodies, having affinities of binding to TNF $\alpha$  of at least  $1 \times 10^8 \text{ M}^{-1}$ , more 20 preferably at least  $1 \times 10^9 \text{ M}^{-1}$  (expressed as Ka) are preferred for immunoassays which detect very low levels of TNF in biological fluids. In addition, anti-TNF antibodies having such high affinities are preferred for therapy of TNF- $\alpha$ -mediated conditions or pathology states.

25 The specificity of cA2 for TNF was confirmed by testing for cross-neutralization of human lymphotoxin (TNF- $\beta$ ). Lymphotoxin shares some sequence homology and certain biological activities, for example, tumor cell cytotoxicity, with TNF (Pennica, et al., Nature 312:724-729 (1984)). 30 Cultured human A673 cells were incubated with increasing concentrations of human lymphotoxin (GENENTECH, San Francisco, CA) with or without 4  $\mu\text{g}/\text{ml}$  chimeric A2 in the presence of 20  $\mu\text{g}/\text{ml}$  cycloheximide at 39°C overnight. Cell death was measured by vital staining with naphthol 35 blue-black, as above. The results indicated that cA2 was

-48-

ineffective at inhibiting and/or neutralizing human lymphotoxin, confirming the TNF $\alpha$ -specificity of the chimeric antibody.

The ability of A2 or cA2 to react with TNF from 5 different animal species was also evaluated. As mentioned earlier, there are multiple epitopes on human TNF to which inhibiting and/or neutralizing mAbs will bind (Moller, et al., *infra*). Human TNF has bioactivity in a wide range of host animal species. However, certain inhibiting and/or 10 neutralizing epitopes on human TNF are conserved amongst different animal species and others appear to be restricted to humans and chimpanzees.

Neutralization experiments utilized endotoxin-activated cell supernatants from freshly isolated human, chimpanzee, 15 rhesus and cynomolgus monkey, baboon, pig, dog, rabbit, or rat monocytes as the TNF source. As discussed above, murine mAb A2 inhibited or neutralized activity of only human and chimpanzee TNF, and had no effect on TNF derived from other primates and lower animals. A2 also did not inhibit or 20 neutralize the cytotoxic effect of recombinant mouse TNF.

Thus, the epitope recognized by A2 is one shared by human and chimpanzee TNF $\alpha$ . Chimeric A2 was also tested in this manner for cross-reactivity with monocyte-derived TNF from rat, rabbit, dog and pig, as well as with purified 25 recombinant mouse TNF $\alpha$ , and natural and recombinant human TNF $\alpha$ . Chimeric A2 only inhibited or neutralized natural and recombinant human TNF $\alpha$ . Therefore, cA2 appears to share species specificity with murine A2.

**EXAMPLE XI - In Vitro Activity and Neutralization Efficacy 30 of a Chimeric Anti-TNF Antibody**

Both the murine and chimeric anti-TNF $\alpha$  antibodies, A2 and cA2 were determined to have potent TNF-inhibiting and/or neutralizing activity. In the TNF cytotoxicity assay described above, murine A2, at a concentration of about

-49-

125 ng/ml completely inhibited or neutralized the biological activity of a 40 pg/ml challenge of rhTNF $\alpha$ . Two separate determinations of inhibiting and/or neutralizing potency, expressed as the 50% Inhibitory Dose (ID50) were determined 5 to be 15.9  $\pm$  1.01 and 17.9  $\pm$  1.6 ng/ml (Mean  $\pm$  Std error). Thus the mAb A2 has an ID50 of about 17 ng/ml.

In this same experimental system, three other murine anti-TNF $\alpha$  antibodies (termed TNF-1, TNF-2 and TNF-3) of comparable binding affinity to TNF were found to have ID50 10 values of 1-2 orders of magnitude greater, and thus were significantly less potent in neutralization than A2.

The ability of cA2 to inhibit or neutralize human TNF $\alpha$  bioactivity in vitro was tested using the bioassay system described above. Cultured A673 cells were incubated with 15 40 pg/ml natural (Genzyme, Boston, MA) or recombinant (Suntory, Osaka, Japan) human TNF with or without antibody overnight as above, and cell death was measured by vital staining. As expected based upon the above results with the A2 mouse mAb, cA2 also inhibited or neutralized both natural 20 and rhTNF in a dose-dependent manner in the cytotoxicity assay. In this assay format, levels of cA2 as low as 125 ng/ml completely abolished the toxic activity of TNF. Upon repeated analysis, the cA2 exhibited greater TNF-inhibiting and/or neutralizing activity than did the parent murine A2 25 mAb. Such inhibiting and/or neutralizing potency, at antibody levels below 1  $\mu$ g/ml, can easily be attained in the blood of a subject to whom the antibody is administered. Accordingly, such highly potent inhibiting and/or 30 neutralizing anti-TNF antibodies, in particular the chimeric antibody, are preferred for therapeutic use in TNF $\alpha$ -mediated pathologies or conditions.

As mentioned above, TNF induces cellular secretion of IL-6. Furthermore, there is evidence that IL-6 is involved in the pathophysiology of sepsis, although the precise role 35 of IL-6 in that syndrome is unclear (Fong, et al., J. Exp.

-50-

Med. 170:1627-1633 (1989); Starnes Jr., et al., *J. Immunol.* 145:4185-4191 (1990)). The ability of cA2 to inhibit or neutralize TNF-induced IL-6 secretion was evaluated using cultured human diploid FS-4 fibroblasts. The results in 5 Table 1 show that cA2 was effective in blocking IL-6 secretion in cells that had been incubated overnight with TNF. TNF-induced IL-6 secretion was not inhibited in the absence of a mAb or in the presence of a control mAb specific for an irrelevant antigen.

TABLE 1 In Vitro Neutralization of TNF-Induced IL-6 Secretion

Antibody	TNF Concentration (ng/ml)			
	0	0.3	1.5	7.5
None	<0.20	1.36	2.00	2.56
Control mAb	<0.20	1.60	1.96	2.16
cA2	<0.20	<0.20	<0.20	0.30

Values represent mean concentrations of IL-6 of duplicate wells, in ng/ml. RhTNF (Suntory, Osaka, Japan), with or without 4  $\mu$ g/ml antibody, was added to cultures of FS-4 fibroblasts and after 18 h, the supernatant was assayed for IL-6 using the QUANTIKINE Human IL-6 Immunoassay (from R&D Systems, Minneapolis, MN). Control mAb = chimeric mouse/human IgG1 anti-platelet mAb (7E3).

The ability of TNF to activate procoagulant and adhesion molecule activities of endothelial cells (EC) is thought to be an important component of pathology pathophysiology. In particular, this can be associated with 5 the vascular damage, disseminated intravascular coagulation, and severe hypotension that is associated with the sepsis syndrome. Therefore, the ability of cA2 to block TNF-induced activation of cultured human umbilical vein endothelial cells (HUVEC) was evaluated. TNF stimulation of

-51-

procogulant activity was determined by exposing intact cultured HUVEC cells to TNF (with or without antibody) for 4 hours and analyzing a cell lysate in a human plasma clotting assay. The results in Table 2 show the expected 5 upregulation by TNF of HUVEC procogulant activity (reflected by a decreased clotting time). Chimeric antibody cA2 effectively inhibited or neutralized this TNF activity in a dose-dependent manner.

TABLE 2 In Vitro Neutralization of TNF-Induced Procogulant Activity

Antibody	$\mu\text{g}/\text{ml}$	TNF Concentration (ng/ml)		
		250	25	0
None	-	64 $\pm$ 4*	63 $\pm$ 1	133 $\pm$ 13
Control Ab	10.00	74 $\pm$ 6	N.D.	178 $\pm$ 55
cA2	10.00	114 $\pm$ 5	185 $\pm$ 61	141 $\pm$ 18
cA2	3.30	113 $\pm$ 2	147 $\pm$ 3	N.D.
cA2	1.10	106 $\pm$ 1	145 $\pm$ 8	N.D.
A2	0.37	73 $\pm$ 17	153 $\pm$ 4	N.D.
cA2	0.12	64 $\pm$ 1	118 $\pm$ 13	N.D.

\* Values represent mean plasma clotting time, in seconds ( $\pm$  S.D.). Clotting time was determined in normal human plasma after addition of the rhTNF (Dainippon, Osaka, Japan) with or without antibody-treated HUVEC lysate and  $\text{Ca}^{++}$  at 37°C. N.D. = not done. Control Ab is a chimeric mouse/human IgG1 anti-CD4 antibody.

In addition to stimulating procogulant activity, TNF also induces surface expression of endothelial cell adhesion molecules such as ELAM-1 and ICAM-1. The ability of cA2 to inhibit or neutralize this activity of TNF was measured 5 using an ELAM-1 specific detection radioimmunoassay. Cultured HUVEC were stimulated with 250 ng/ml rhTNF (Dainippon, Osaka, Japan) with or without antibody at 37°C

-52-

overnight in a 96-well plate format. Surface expression of ELAM-1 was determined by sequential addition of a mouse anti-human ELAM-1 mAb and  $^{125}\text{I}$ -labelled rabbit anti-mouse immunoglobulin second antibody directly to culture plates at 5 4°C.

TNF induced the expression of ELAM-1 on the surface of cultured HUVEC cells, and this activity was again effectively blocked in a dose-related manner by cA2.

Finally, TNF is known to stimulate mitogenic activity 10 in cultured fibroblasts. Chimeric A2 inhibited or neutralized TNF-induced mitogenesis of human diploid FS-4 fibroblasts cultures, confirming the potent inhibiting and/or neutralizing capability of cA2 against a broad spectrum of in vitro TNF biological activities.

15 EXAMPLE XII - Determination of amino acid sequences (epitope) on human TNF- $\alpha$  recognized by cA2 mAb

Reagents

The following reagents are readily available from commercial sources. FMOC-L-Ala-OPfp, FMOC-L-Cys(Trt)-OPfp, 20 FMOC-L-Asp(OtBu)-OPfp, FMOC-L-Glu(OtBu)-OPfp, FMOC-L-Phe-OPfp, FMOC-Gly-OPfp, FMOC-L-His(Boc)-OPfp, FMOC-L-Ile-OPfp, FMOC-L-Lys(Boc)-OPfp, FMOC-L-Leu-OPfp, FMOC-L-Asn-OPfp, FMOC-L-Pro-OPfp, FMOC-L-Gin-OPfp, FMOC-L-Arg(Mtr)-OPfp, FMOC-L-Ser(tBu)-ODhbt, 25 FMOC-L-Thr(tBu)-ODhbt, FMOC-L-Val-OPfp, FMOC-L-Trp-OPfp, FMOC-L-Try(tBu)-OPfp, and 1-hydrox-fbenotriazol (HOBT) were obtained from Cambridge Research Biochemicals. Piperidine and was obtained from Applied Biosystems, Inc. 1-Methyl-2-Pyrrolidinone (NMP) was obtained from EM Science; 30 Methanol from JT Baker; Acetic Anhydride from Applied Biosystems, Inc., Trifluoroacetic acid (TFA) from Applied Biosystems, Inc.; Diisopropylamine (DIEA), Triethylamine, Dithiothreitol (DTT) and Anisole from Aldrich and Hydrochloric Acid (HCl) from JT Baker.

-53-

Abbreviations: FMOC, 9-fluorenylmethoxycarbonyl; tBu t-butyl ether; OrB, t-butyl ester; Boc, t-butyloxycarbonyl; Mtr, 4-methoxy-2,3,6-trimethylbenzenesulfonyl; Trt, trityl; OPfp, pentafluorophenylester; 5 ODhbt. oxo-benzotriazole ster.

A chimeric antibody of the present invention, designated cA2, was used to determine which portions of the TNF amino acid sequence were involved in inhibitory binding by the antibody by epitope mapping, whereby the amino acid 10 sequences of TNF- $\alpha$  recognized by cA2 have been identified.

The complete primary sequence of human TNF $\alpha$  is disclosed in *Nature* 312:724-729 (1984). Overlapping decapeptides beginning with every second amino acid and covering the entire amino acid sequence of human TNF- $\alpha$  were 15 synthesized on polyethylene pins using the method of Gysen (Gysen et al., *Peptides: Chemistry and Biological, Proceedings of the Twelfth American Peptide Symposium*, p. 519-523, Ed, G.R. Marshall, Escom, Leiden, 1988). Sets of peptide pins bearing free N-terminal amino groups and 20 acetylated N-terminal amino groups were individually prepared. Both sets of peptide pins were incubated in solutions containing the anti-TNF mAb cA2 to determine the amino acid sequences that make up the cA2 epitope on human TNF- $\alpha$ , as described below. The O.D. (optional density) 25 correlates directly with the increased degree of cA2 binding. This competitive binding study delineates peptides which can show non-specific binding to cA2.

There are at least two non-contiguous peptide sequences of TNF- $\alpha$  recognized by cA2. Using the conventional protein 30 numbering system wherein the N-terminal amino acid is number 1, the cA2 mAb recognizes an epitope composed at least in part of amino acids located within residues 87-108 or both residues 59-80 and 87-108 of TNF.

Unexpectedly, the mAb cA2 blocks the action of TNF- $\alpha$  35 without binding to the putative receptor binding locus,

-54-

which can include one or more of, e.g., 11-13, 37-42, 49-57 or 155-157 of hTNF $\alpha$ . Preferred anti-TNF mAbs are those that inhibit this binding of human TNF- $\alpha$  to its receptors by virtue of their ability to bind to one or more of these 5 peptide sequences. These antibodies can block the activity of TNF by virtue of binding to the cA2 epitope, such binding demonstrated to inhibit TNF activity. The identification of those peptide sequences recognized by cA2 provides the information necessary to generate additional mAbs with 10 binding characteristics and therapeutic utility that parallel the embodiments of this application.

#### **Peptide Pin Synthesis**

Using an epitope mapping kit purchased from Cambridge Research Biochemicals, Inc. (CRB), dodecapeptides 15 corresponding to the entire sequence of human TNF- $\alpha$  were synthesized on polyethylene pins.

A synthesis schedule was generated using the CRB epitope mapping software. Prior to the first amino acid coupling, the pins were deprotected with a 20% piperidine in 20 NMP solution for 30 minutes at room temperature. After deprotected, the pins were washed with NMP for five minutes at room temperature, followed by three methanol washes. Following the wash steps, the pins were allowed to air dry for at least 10 minutes.

25 The following procedure was performed for each coupling cycle:

- 1) The amino acid derivatives and the HOBT were weighted out according to the weights required in the synthesis schedule.
  
- 30 2) The HOBT was dissolved in the appropriate amount of NMP according to the synthesis schedule.

-55-

3) The amino acid derivatives were dissolved in the recommended amount of HOBT solution and 150 microliters were pipeted into the appropriate wells as directed by the well position sheet of 5 the synthesis schedule.

4) The blocks containing the pins were placed into the wells, and the "sandwich" units stored in plastic bags in a 30°C water bath for 18 hours.

5) The pins were removed from the wells and washed once (for 5 minutes) with NMP, three times (for 10 minutes) with methanol and air dried for 10 minutes.

10 6) The pins were deprotected as described above and the procedure repeated.

15 To acetylate the peptides on one block of pins, the peptide pins were washed, deprotected and treated with 150 microliters of a solution containing NMP; acetic anhydride:triethylamine (5:2:1) for 90 minutes at 30°C, followed by the washing procedure outlined above. The 20 second set of peptide pins was deprotected by not acetylated to give free N-terminal amino groups.

The final deprotection of the peptides to remove the side chain protecting groups was done using a mixture of TFA:anisole:dithiothreitol, 95:2.5:2.5 (v/v/w) for four 25 hours at ambient temperature. After deprotection, the pins were air dried for 10 minutes, followed by a 15 minute sonication in a solution of 0.1% HCl in methanol/distilled water (1:1). The pins dried over night and were then ready for testing.

-56-

**ELISA Assay for cA2 Binding to TNF- $\alpha$  Peptide PINs**

**Reagents: Disruption Buffer**

Sodium dihydrogen phosphate (31.2 g, Sigma cat # S-0751 or equivalent) and sodium dodecylsulfate (20.0 g, Sigma cat 5 # L-3771 or equivalent) were dissolved in 2.0 L of milliQ water. The pH was adjusted to 7.2  $\pm$  0.1 with 50% w/w sodium hydroxide (VWR cat # VW6730-3 or equivalent).

**Blocking Buffer**

Sodium dihydrogen phosphate (0.39 g, Sigma cat #S-0751 10 or equivalent) disodium hydrogen phosphate (1.07 g, Baker cat # 3828-1 or equivalent) and sodium chloride (8.50 g, Baker cat # 3624-5 or equivalent were dissolved in 1.0 L of milliQ water. The pH was adjusted to 7.2  $\pm$  0.1 with 50% w/w sodium hydroxide (VWR cat VW6730-3 or equivalent). Chicken 15 egg albumin (10.0 g, Sigma cat #A-5503 or equivalent) and bovine serum albumin (10.0 g, Sigma, cat #A-3294 or equivalent) were dissolved at room temperature with gentle stirring. The solution was filtered, and to the solution was added Tween 20 (2.0 ml, Sigma cat #P-13.79 or 20 equivalent). The solution was stirred gently at room temperature for 30 min, filtered and stored at 40°.

**PBS/Tween 20**

A 10 x concentrate was prepared by dissolving sodium dihydrogen phosphate (3.90 g, Sigma cat # S-0751 or 25 equivalent), disodium hydrogen phosphate (10.70 g, Baker cat #3828-1 or equivalent) and sodium chloride (85.0 g, Baker cat #3624-5 or equivalent) in 1.0 L of milliQ water. The pH was adjusted to 7.2  $\pm$  0.1 with 50% w/w sodium hydroxide (VWR cat #VW 6730 or equivalent). To the solution was added 30 Tween 20 (5.0 mL, Sigma cat #P-1379 or equivalent), and the mixture stirred gently. Just prior to use 100 mL of this solution was diluted to 1.0 L with milliQ water.

-57-

#### Substrate solution

Substrate buffer was prepared by dissolving citric acid (4.20g, Malinckrodt cat #0627 or equivalent) and disodium hydrogen phosphate (7.10 g, Baker cat #3828-1 or equivalent) 5 in 1.0 L of milliQ water. The pH was adjusted to 5.00 with 50% w/w sodium hydroxide (VWR cat #VW6730-3 or equivalent). Immediately prior to use an OPD substrate tablet (30 mg, Sigma cat #P-8412 or equivalent and 30% (v/v) hydrogen peroxide (40  $\mu$ L, Sigma cat #P-1379 or equivalent) were added 10 to the substrate buffer 25.0 mL).

The solution was wrapped in foil and mixed thoroughly.

#### 4 NH<sub>2</sub>SO<sub>4</sub>

Sulfuric acid (53 mL, EM Science cat #SX1244-5 or equivalent) was slowly added to MILLI-Q water (447 mL) and 15 cooled to room temperature prior to use.

#### Equipment

Molecular Devices Model nu-max plate reader or equivalent. Scientific Products Model R4140 Oscillating table shaker and equivalent. BRANSON Model 5200 ultra-sonic 20 bath or equivalent. FINNPIPETTE Model 4172317 multichannel pipeter or equivalent. CORNING Model 25801 96 well disposable polystyrene Elisa Plates.

Prior to use and after each subsequent use the peptide pins were cleaned using the following procedure. Disruption 25 buffer (2.0 L) was heated to 60° and placed in an ultra-sonic bath in a fume hood. To the disruption buffer was added dithioltreitol (2.5 g, Sigma cat #D-0632 or equivalent). The peptide pins were sonicated in this medium for 30 min, washed thoroughly with milliQ waster, suspended 30 in a boiling ethanol bath for 2 min, and air-dried.

Blocking buffer (200  $\mu$ L) was added to a 96 well disposable polystyrene Elisa plate and the peptide pins suspended in the wells. The peptide pins and plate were

-58-

incubated for 2 hours at room temperature on an oscillating table shaker. The plates and peptide pins were washed with PBS/Tween 20 (four times). To each well was added a 20  $\mu$ g/ml concentration of cA2 antibody (diluted with 5 blocking buffer, 175  $\mu$ L/well). TNF competition was done by incubation of TNF $\alpha$  (40  $\mu$ g/ml) and cA2 (20  $\mu$ g/ml) in BSA/ovalbumin/ BBS for three hours at room temperature. The peptide pins were suspended in the plate and incubated at 4° overnight. The peptide pins and plate were washed with 10 PBS/Tween 20 (four times). To each well was added anti-human goat antibody conjugated to horseradish peroxidase (diluted with blocking buffer to 1/2000, 175  $\mu$ L/well, Jackson IMMUNORESEARCH Labs). The peptide pins were suspended in the plate, and incubated for 1 hour at 15 room temperature on a oscillating table shaker. The plates and peptide pins were washed with PBS/Tween 20 (four times). To each well added freshly prepared substrate solution (150  $\mu$ L/well), the peptide pins were suspended in the plate and incubated for 1 hour at room temperature on an 20 oscillating table shaker. The peptide pins were removed and to each well is added 4N H<sub>2</sub>SO<sub>4</sub> (50  $\mu$ L). The plates were read in a Molecular Devices plate reader (490 nm, subtracting 650 nm as a blank).

**EXAMPLE XIII - Production Mouse Anti-Human TNF mAb Using TNF 25 Peptide Fragments**

Female BALB/c mice, as in Example I above, are injected subcutaneously and intraperitoneally (i.p.) with forty  $\mu$ g of purified *E. coli*-derived recombinant human TNF (rhTNF) fragments comprising anti-TNF epitopes of at least 5 amino 30 acids located within the non-contiguous sequence 59-80, 87-108 or both residues 59-80 and 87-108 of TNF (of SEQ ID NO:1), as presented above, emulsified with an equal volume of complete Freund's adjuvant (obtained from Difco Laboratories) in 0.4 ml is into a mouse. One week later, a

-59-

booster injection of 5  $\mu$ g of these rhTNF fragments in incomplete Freund's adjuvant is given i.p. followed by four consecutive i.p. injections of 10  $\mu$ g of TNF fragments including anti-TNF epitopes including amino acids from 5 residues 59-80, 87-108 or both 59-80 and 87-108 of hTNF $\alpha$  (of SEQ ID NO:1) without adjuvant. Eight weeks after the last injection, the mouse is boosted i.p. with 10  $\mu$ g of TNF.

Four days later, the mouse is sacrificed, the spleen is obtained and a spleen cell suspension is prepared. Spleen 10 cells are fused with cells of the nonsecreting hybridoma, Sp2/0 (ATCC CRL1581), at a 4:1 ratio of spleen cells to Sp2/0 cells, in the presence of 0.3 ml of 30% polyethylene glycol, PEG 1450. After incubation at 37°C for 6 hours, the fused cells are distributed in 0.2 ml aliquots into 96-well 15 plates at concentrations of  $2 \times 10^4$  SP2/0 cells per well. Feeder cells, in the form of  $5 \times 10^4$  normal BALB/c spleen cells, are added to each well.

The growth medium used consisted of RPMI-1640 medium, 10% heat-inactivated fetal bovine serum (FBS) (Hyclone), 20 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin (GIBCO Laboratories) and, for selection, hypoxanthine-aminopterin-thymidine (HAT) (Boehringer Mannheim). A solid-phase radioimmunoassay (RIA) is employed for screening 25 supernatants for the presence of mAbs specific for rhTNF $\alpha$  fragments including portions of residues 59-80, 87-108 or both 59-80 and 87-108 of hTNF $\alpha$  (of SEQ ID NO:1). This assay is described in Example II, above. The background binding in this assay is about 500 cpm. A supernatant is considered 30 positive if it yielded binding of 2000 cpm or higher.

Of the supernatants screened, one or more positive supernatants are routinely identified by RIA. Of these positive supernatants, the highest binding (as shown by the higher cpm values) are subcloned at limiting dilution on 35 mouse feeder cells. Upon further analysis of the

-60-

supernatants in neutralization assays, routinely one or more antibodies are found to have potent inhibiting and/or neutralizing activity. These positive and inhibiting and/or neutralizing hybridoma lines are then selected and 5 maintained in RPMI-1640 medium with 10% FBS (GIBCO), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

**EXAMPLE XIV - Production of Murine and chimeric antibodies, fragments and regions from TNF Peptides**

10 Murine and chimeric antibodies, fragments and regions are obtained by construction of chimeric expression vectors encoding the mouse variable region of antibodies obtained in Example XIV and human constant regions, as presented in Examples IV-IX above.

15 The resulting chimeric A2 antibody is purified from tissue culture supernatant by Protein A-Sepharose chromatography. The supernatant is adjusted to 0.1M Tris, 0.002M EDTA, pH 8.0 and loaded on a Protein A-Sepharose column equilibrated in the same buffer. The IgG is then 20 eluted with 0.1M citrate, pH 3.5, neutralized with 1M Tris, and dialyzed into phosphate buffered saline (PBS).

The purified murine and chimeric antibodies, fragments and regions are evaluated for its binding and inhibiting and/or neutralizing activity.

25 MULTIPLE ADMINISTRATION OF cA2 TO PATIENTS SUFFERING FROM RHEUMATOID ARTHRITIS

The retreatment program included patients 1, 2, 3, 5, 7, 8, 9 and 10 suffering from rheumatoid arthritis from an open-label trial with cA2. Patients received up to four 30 cycles of treatment, the complete infusion protocol in the open trial being cycle 1. The timing of cycles 2-4 was dictated by disease relapse, defined as the loss of response

-61-

to the previous cycle. Most cycles were administered within 5 weeks of relapse, but cycle 2 was delayed in 4 patients for administrative reasons. Although 8 patients entered the retreatment program, patient 5 was excluded from the 5 analysis of response because of withdrawal for an adverse event during cycle 2 (see below). Three patients were withdrawn after cycle 2. The response data therefore derive from 7 patients for cycles 1 and 2, 5 for cycle 3, and 4 for cycle 4. The total periods of observation, including 10 periods of disease relapse, varied from 17 to 108 weeks.

CA2 was administered by intravenous infusion over 2-3 hours. The dose was 30 mg/kg in cycle 1 (in two or four infusions) and 10 mg/kg in a single infusion for cycles 2-4. Patients were admitted overnight for cycle 1, but 15 subsequently were treated as day cases.

Other drugs were maintained at stable dose from the beginning of cycle 1, except for some alterations after disease relapse between cycles 1 and 2: patient 3 took an increased dose of prednisolone for 1 week; patient 5 received 20 a single intra-articular injection of methylprednisolone; and patients 8 and 10 ceased their non-steroidal anti-inflammatory drugs. As in the open trial, additional steroids by any route were forbidden during the study, but simple analgesics were freely allowed.

25 The primary measure of response was the multi-variable Paulus index, calculated at two levels (Paulus 20% and 50%) and modified to accommodate the format of the data collected. Laboratory measurements included the erythrocyte sedimentation rate (ESR, Westergren), C-reactive protein 30 (CRP, rate nephelometry), and autoantibodies measured as described. Human anti-chimeric A2 antibody responses (HACAs) were measured with a double-antigen enzyme immunoassay. False-positive signals due to rheumatoid factor anti-Fc antibodies were eliminated by the addition of 35 covalently polymerized human Fc to the HACA sample diluent.

-62-

Samples containing over 200 ng/mL cA2 (independent assay for free cA2) were considered likely to give a false-negative result for HACA, and were disqualified from analysis.

Each patient achieved a response to treatment cycle 1 and showed repeated responses after cycles 2-4, with maintenance of the response magnitude. The median maximum improvement in individual disease-activity assessments, such as the swollen-joint count and CRP, exceeded 80% after each cycle (data not shown). The median (interquartile range) swollen-joint counts before and the best assessment after each cycle were: (before, after) cycle 1, 21 (16-25), 3 (0-3) ( $p=0.011$  by Wilcoxon's signed-ranks test); cycle 2, 16 (8-21), 2 (0-4) ( $p=0.011$ ); cycle 3, 8 (6.5-18.5), 1 (1-3) ( $p=0.03$ ); and cycle 4, 11 (10.3-14.8), 2 (2-6.5) ( $p>0.05$ ). Equivalent data for CRP were: cycle 1, 31 mg/L (10-44, normal <10 mg/L), 0 (0-5) ( $p=0.011$ ); cycle 2, 49 (24-62), 3 (2-7) ( $p=0.011$ ); cycle 3, 39 (24-69.5), 0 (0-17..5) ( $p=0.03$ ); and cycle 4, 40.5 (11.5-125), 5 (0-65.5) ( $p>0.05$ ).

The overall pattern of response in a patient who completed all four cycles is shown in Figure 1. As shown, there was a co-ordinated change in swollen-joint count and CRP.

Analysis of possible changes in the duration of the response was complicated by the dose reduction in cycles 3-4 and the change from a multiple-divided-dose infusion in cycle 1 to a single infusion later. Individual patients showed varying response patterns (Figure 2) but overall, the duration tended to shorten with successive cycles. The median Paulus 20% response duration after treatment with 20 mg/kg cA2 in cycle 1 was 12 weeks (interquartile range 8-17.4). Equivalent values for cycles 2-4 (when patients received half this dose) were 9.1 weeks (1-19.1), 8.3 weeks (3.2-12.5), and 7.7 weeks (1.6-15.2) ( $p>0.05$  compared with cycle 1 by Friedman's test, repeated Wilcoxon's signed-ranks tests, and linear regression).

-63-

Forty-one infusions were administered in this study and were well tolerated, with 2 exceptions. Patient 5 was withdrawn after the administration of only 1% of the scheduled cA2 dose in cycle 2. This followed an episode of 5 vasovagal syncope, consequent on a traumatic venepuncture. Patient 9 developed fever, headache, and transient facial flushing during cycle 4, but was treated symptomatically and not withdrawn. Other adverse events that were considered reasonably related to cA2 are outlined in the table. Three 10 events prompted the precaution of early withdrawal (patients 1, 7 and 10).

TABLE 3: Adverse Events

Patient	Event	Time*	Relation to cA2
1	Urticaria	20/1 (C2)	Possibly
2	Anti-nuclear antibodies	48/4 (C3)	Possibly
3	Pruritis	18/during (C3)	Possibly
5	Vasovagal Syncope	16/during (C2)	Possibly
7	Chronic sinusitis	25/25 (C1)	Possibly
8	Eczema Pharyngitis	32/13 (C2) 51/2 (C4)	Possibly
9	Urinary Tract Infection Anti-nuclear antibodies Flushing, headache, fever (38°C)	58/1 (C1) 61/4 (C2) 95/during (C4)	Possibly Probably Probably
10	dsDNA and cardiolipin antibodies	6/6 (C1)	Probably

\* Weeks after cycle 1/weeks after last cycle: C=last cycle number. dsDNA=double-stranded DNA. Only events considered reasonably related to cA2 included. All events resolved completely, except for eczema in patient 8 and laboratory changes in 9.

-64-

Four patients had no HACA responses when tested at least 6 weeks after the last infusion. The remaining 4 patients developed HACAs at varying times after retreatment (titres 10, 20, 80 and 640 in patients 3, 1, 5 and 9, 5 respectively), all specific for the murine variable region of cA2. Of these, 2 patients completed all four cycles, 1 completed two cycles and 1 was withdrawn during cycle 2. Some patients with HACAs showed a reduction in response duration in cycles 2-4. In other patients, however, no 10 clear relation was evident. Patient 9 developed a high-titre HACA (640) after cycle 2, but her cycle 3 response duration of 8.7 weeks was no different from her cycle 1 duration (8 weeks). Conversely, no HACA was detected in patient 8, but her response duration fell from 17.4 weeks in 15 cycle 1 to 8.3 in cycle 3.

The data show that patients with flares of rheumatoid arthritis can be successfully managed with cA2, which provides an alternative to traditional treatments such as hospital admission, high-dose corticosteroids, or cytotoxic 20 therapy. The requirement for disease relapse before retreatment represented a difficult therapeutic challenge. Despite this, a response was achieved after each patient/cycle, with impressive improvements in clinical and laboratory measures of inflammation. The success in 25 demonstrating repeated responses in the same individuals suggests that regular treatment with cA2 can achieve long-term disease suppression.

The adverse events included the development of 30 antinuclear antibodies in 3 of 7 patients. Although two of these findings were not associated with specific autoantibodies, patient 10 developed significant titres of dsDNA and cardiolipin antibodies after cycle 1, and showed a further rise in titres after cycle 2. Although no clinical features of systemic lupus erythematosus developed, she was 35 withdrawn from the study. A cautious approach was adopted

-65-

to minor infective events, withdrawing patient 7 after the development of sinusitis. With more experience in the repeated use of cA2, a lower dropout rate may be achievable.

Only 1 of 20 patients in the original open-label trial 5 developed an antiglobulin response, suggesting that cA2 is not especially immunogenic. HACAs specific for the murine portion of cA2 were eventually detected in half of the patients in the retreatment program. These were mostly low titre and 2 patients were successfully retreated despite 10 their presence. Similar antiglobulin responses were seen in 3 of 4 rheumatoid arthritis patients treated with repeated injections of a humanized monoclonal antibody to CD252, suggesting that antibody reshaping does not entirely eliminate immunogenicity.

15 Equivalents

Those skilled in the art will know, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other equivalents are 20 intended to be encompassed by the following claims.

-66-

CLAIMS

1. A method of treating or preventing reoccurrence of a TNF-mediated disease in an individual having the TNF-mediated disease comprising administering multiple doses of an anti-TNF antibody wherein the second or subsequent dose is administered during or prior to reoccurrence of the disease.  
5
2. The method of Claim 1 wherein the TNF-mediated disease is selected from the group consisting of autoimmune disease, acute or chronic immune disease, bacterial infection, viral infection, parasitic infection, inflammatory disease, neurodegenerative disease, malignancy and alcohol-induced hepatitis.  
10
3. The method of Claim 2 wherein the anti-TNF antibody is a monoclonal antibody or fragment thereof having low immunogenicity.  
15
4. The method of Claim 3 wherein the anti-TNF antibody has a high affinity for TNF $\alpha$ .  
20
5. The method of Claim 4 wherein the antibody is selected from the group consisting of a chimeric antibody, a humanized antibody or a resurfaced antibody or fragment thereof.  
25
6. The method of Claim 5 wherein the antibody binds to one or more amino acids of hTNF $\alpha$  selected from the group consisting of about 87-108 and about 59-80.  
25
7. The method of Claim 5 wherein the antibody binds to the epitope of A2.  
25

-67-

8. The method of Claim 5 wherein the antibody is a chimeric antibody.
9. The method of Claim 8 wherein the antibody binds to one or more amino acids of hTNF $\alpha$  selected from the group consisting of about 87-108 and about 59-80.  
5
10. The method of Claim 8 wherein the antibody binds to the epitope of cA2.
11. The method of Claim 10 wherein the antibody is cA2.
12. A method of treating or preventing reoccurrence of rheumatoid arthritis in an individual in need thereof comprising administering to the individual multiple doses of an anti-TNF $\alpha$  antibody wherein the second or subsequent dose is administered during or prior to reoccurrence of the disease.  
10
13. The method of Claim 12 wherein the anti-TNF antibody is a monoclonal antibody or fragment thereof having low immunogenicity.  
15
14. The method of Claim 13 wherein the antibody is selected from the group consisting of a chimeric antibody, a humanized antibody or a resurfaced antibody or fragment thereof.  
20
15. The method of Claim 14 wherein the antibody binds to one or more amino acids of hTNF $\alpha$  selected from the group consisting of about 87-108 and about 59-80.
16. The method of Claim 14 wherein the antibody binds to the epitope of A2.  
25

-68-

17. The method of Claim 14 wherein the antibody is a chimeric antibody.
18. The method of Claim 17 wherein the antibody binds to one or more amino acids of hTNF $\alpha$  selected from the 5 group consisting of about 87-108 and about 59-80.
19. The method of Claim 17 wherein the antibody binds to the epitope of cA2.
20. The method of Claim 19 wherein the antibody is cA2.
21. A method of treating a TNF-mediated disease in an 10 individual having the TNF-mediated disease and treating or preventing reoccurrence of the TNF-mediated disease comprising administering multiple doses of an anti-TNF antibody.
22. An anti-TNF antibody for use in the manufacture of a 15 medicament for multiple administration in the treatment or prevention of reoccurrence of a TNF-mediated disease.
23. The anti-TNF antibody of Claim 22 wherein the antibody is a monoclonal antibody or fragment thereof having low immunogenicity and high affinity for TNF $\alpha$ .
- 20 24. The anti-TNF antibody of Claim 23 wherein the antibody is a chimeric antibody.
25. The anti-TNF antibody of Claim 24 wherein the chimeric antibody binds to the epitope of cA2.
26. The anti-TNF antibody of Claim 25 wherein the chimeric 25 antibody is cA2.

1/2

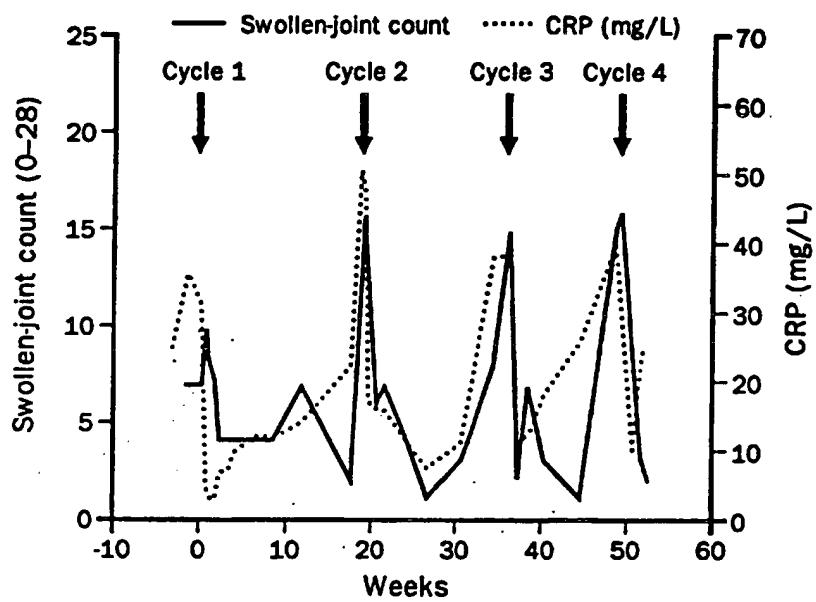


FIGURE 1

2/2

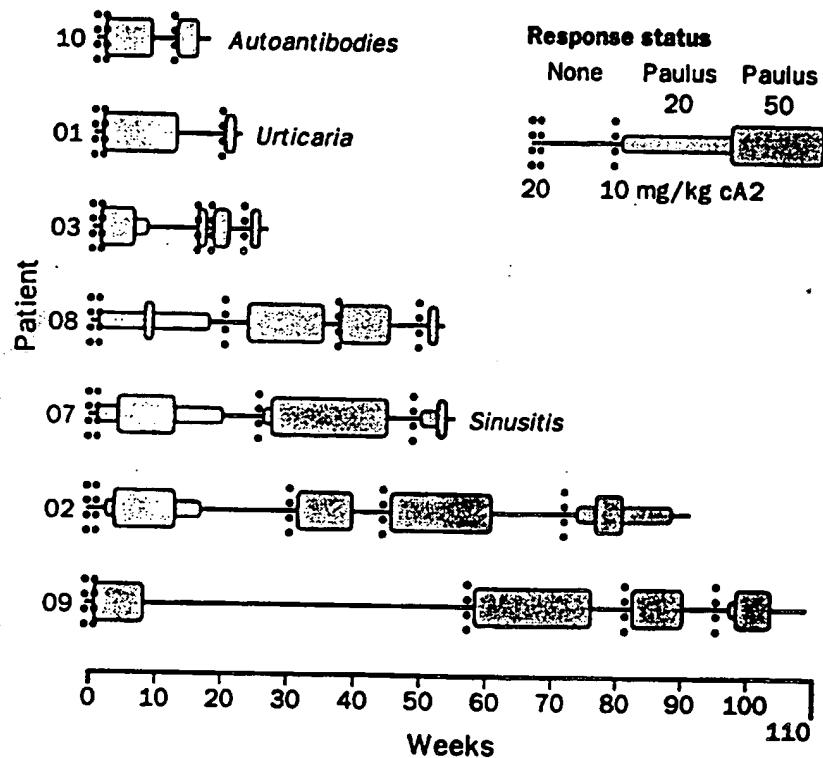


FIGURE 2

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/05155

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/00; C07K 14/525, 16/24, 16/42, 16/46; A69K 39/395

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/134.1, 136.1, 158.1, 198.1, ; 435/69.6, 70.21, 172.2, 240.27, ; 530/387.2, 387.3, 388.23, 389.2; 536/23.51; 935/15

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 9102078, (RATHJEN ET AL), 21 FEBRUARY 1991, SEE ENTIRE DOCUMENT	1-26
Y	JOURNAL OF EXPERIMENTAL MEDICINE, VOL. 161, ISSUED MAY 1985, BEUTLER ET AL, "PURIFICATION OF CACHECTIN, A LIPOPROTEIN LIPASE-SUPPRESSING HORMONE SECRETED BY ENDOTOXIN-INDUCED RAW 264.7 CELLS" PAGES 984-995, SEE ENTIRE DOCUMENT.	1-26
Y	ISRAEL JOURNAL OF MEDICAL SCIENCES, VOL 28, NO. 2, ISSUED FEBRUARY 1992, ADERKA ET AL, "THE POSSIBLE ROLE OF TUMOR NECROSIS FACTOR (TNF) AND ITS NATURAL INHIBITORS, THE SOLUBLE-TNF RECEPTORS, IN AUTOIMMUNE DISEASES", PAGES 126-130, SEE ENTIRE DOCUMENT.	1-26

Further documents are listed in the continuation of Box C.  See patent family annex.

- \* Special categories of cited documents:
  - "T" later documents published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
  - "A" document defining the general state of the art which is not considered to be of particular relevance
  - "E" earlier document published on or after the international filing date
  - "L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reasons (as specified)
  - "O" document referring to an oral disclosure, use, exhibition or other means
  - "P" document published prior to the international filing date but later than the priority date claimed

Date of the actual completion of the international search	Date of mailing of the international search report
09 JULY 1995	31 JUL 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer T. NISBET Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/05155

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CYTOKINE, VOL. 2, NO. 3, ISSUED MAY 1990, MOLLER ET AL, "MONOCLONAL ANTIBODIES TO HUMAN TUMOR NECROSIS FACTOR $\alpha$ : IN VITRO AND IN VIVO APPLICATION", PAGES 162-169, SEE ENTIRE DOCUMENT.	1-26
Y	JOURNAL OF EXPERIMENTAL MEDICINE, VOLUME 170, ISSUED NOVEMBER 1989, FONG ET AL, "ANTIBODIES TO CACHECTIN/TUMOR NECROSIS FACTOR REDUCE INTERLEUKIN 1 $\beta$ AND INTERLEUKIN 6 APPEARANCE DURING LETHAL BACTEREMIA", PAGES 1627-1633, SEE ENTIRE DOCUMENT.	1-26
Y	ANNALS OF THE RHEUMATIC DISEASES, VOL. 47, ISSUED 19 FEBRUARY 1988, DI GIOVINE ET AL, "TUMOUR NECROSIS FACTOR IN SYNOVIAL EXUDATES", PAGES 768-772, SEE ENTIRE DOCUMENT.	1-26
Y	NATURE, VOL. 330, ISSUED 17 DECEMBER 1987, TRACEY ET AL, "ANTI-CACHECTIN/TNF MONOCLONAL ANTIBODIES PREVENT SEPTIC SHOCK DURING LETHAL BACTERAEMIA", PAGES 662-664, SEE ENTIRE DOCUMENT.	1-26

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/05155

**A. CLASSIFICATION OF SUBJECT MATTER:**  
US CL :

424/134.1, 136.1, 158.1, 198.1, ; 435/69.6, 70.21, 172.2, 240.27, ; 530/387.2, 387.3, 388.23, 389.2; 536/23.51;  
935/15

**B. FIELDS SEARCHED**

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAS, BIOSIS, EMBASE, MEDLINE  
search terms: (antibod? or ig! or immunoglobul?) and (tnf or (tumor(w)necrosis(w)factor?) or cachectin) and (therap? or treat?)

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